

A P P L I C A T I O N  
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on  
METHODS AND COMPOSITIONS FOR DEREPRESSION OF  
IAP-INHIBITED CASPASE

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**METHODS AND COMPOSITIONS FOR DEREPRESSION OF  
IAP-INHIBITED CASPASE**

This application is a continuation-in-part of  
U.S. Application No. 10/302,811, filed November 21, 2002,  
5 which claims benefit of the filing date of U.S.  
Provisional Application No. 60/331,957, filed  
November 21, 2001, both of which are incorporated herein  
by reference.

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Institute of Health/National Cancer Institute. The United  
States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The present invention relates generally to  
15 molecular medicine and more specifically to compositions  
and methods for altering molecular interactions involved  
in regulating programmed cell death.

Normal tissues in the body are formed either by  
cells that have reached a terminally differentiated state  
20 and no longer divide or by cells that die after a period  
of time and are replaced from a pool of dividing cells.  
For example, nervous tissue is formed early in development  
and the cells of the nervous system reach a terminally  
differentiated state soon after birth. In contrast, the  
25 body has a number of self renewing tissues such as skin,  
gut, bone marrow and sex organs which undergo a balanced  
flux of cell birth and death. This flux, which results in  
the production of 50-70 billion cells per day in an

average adult and amounting to a mass of cells equivalent to an entire body weight over a years time, is balanced by the regulated eradication of an equivalent number of cells. In self renewing tissues the eradication is  
5 maintained, in part, due to the process of programmed cell death, known as apoptosis, in which the cells are genetically "programmed" to die after a certain period of time.

Apoptosis is particularly prominent during the  
10 development of an organism, where cells that perform transitory functions are programmed to die after their function no longer is required. In addition, apoptosis can occur in cells that have undergone major genetic alterations, thus providing the organism with a means to  
15 rid itself of defective and potentially cancer forming cells. Apoptosis also can be induced due to exposure of an organism to various external stimuli, including, for example, bacterial toxins, ethanol and ultraviolet radiation. Chemotherapeutic agents for treating cancer  
20 also are potent inducers of apoptosis.

The regulation of programmed cell death is a complex process involving numerous pathways and, on occasion, defects occur in the regulation of programmed cell death. Given the critical role of this process in  
25 maintaining a steady-state number of cells in a tissue or in maintaining the appropriate cells during development of an organism, defects in programmed cell death often are associated with pathologic conditions. It is estimated that either too little or too much cell death is involved  
30 in over half of the diseases for which adequate therapies do not currently exist.

Various disease states occur due to aberrant regulation of programmed cell death in an organism. For example, defects that result in a decreased level of apoptosis in a tissue as compared to the normal level  
5 required to maintain the steady-state of the tissue can result in an increased number of cells in the tissue. Such a mechanism of increasing cell numbers has been identified in various cancers, where the formation of a tumor occurs not because the cancer cells necessarily are  
10 dividing more rapidly than their normal counterparts, but because the cells are not dying at their normal rate.

Thus, a need exists for agents capable of modulating programmed cell death pathways and methods for treating individuals experiencing diseases associated with  
15 aberrant regulation of programmed cell death. The present invention satisfies this need and provides additional advantages as well.

#### **SUMMARY OF THE INVENTION**

The invention provides isolated agents having a  
20 core peptide selected from the group consisting of Core peptides 4 through 39 and 42 through 55, wherein the agent derepresses an IAP-inhibited caspase. Also provided is an isolated agent having a core structure selected from any of the structures shown in Figures 5, 9, 10, 14B, 21-24,  
25 34, 35 and 36, wherein the agent derepresses an IAP-inhibited caspase. The invention further provides a method of derepressing an IAP-inhibited caspase. The method consists of contacting an IAP-inhibited caspase with an effective amount of an agent to derepress an IAP-  
30 inhibited caspase, the agent having a core motif selected

from the group consisting of a core peptide having a sequence set forth in any of Core peptides 4 through 39 and 42 through 55; a core structure selected from the group consisting of TPI759, TPI 882, TPI 914 or TPI 927; 5 and a core structure selected from TPI 1391, TPI 1349, TPI 1396, TPI 1509, TPI 1540, TPI 1400, TPI 792 and TPI 1332. The invention also provides methods for promoting apoptosis in a cell and for reducing the severity of a pathology characterized by reduced levels of apoptosis. 10 Methods for identifying agents that derepress an IAP-inhibited caspase further are provided.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows a plot of values obtained for the 15 ratio of  $V_{\max}$  (where  $V_{\max}$  is equal to RFU/min) for hydrolysis of Acetyl-DEVD-7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC) in the presence and absence of each species of the TPI 1328 library, composed of mixtures of hexapeptides.

20 Figure 2 shows a table listing individual tetrapeptides of the TPI 1313 library and the ratio of  $V_{\max}$  for hydrolysis of Ac-DEVD-AFC in the presence and absence of each peptide species. The ratio = ( $V_{\max}$  when peptide, caspase 3 and XIAP are present)/( $V_{\max}$  when caspase 3 and 25 XIAP are present).

Figure 3 shows structures for the individual species of tetrapeptides in the TPI 1313 library.

Figure 4 shows structures of the defined functionalities in the mixtures found to be derepressors

of an XIAP-inhibited caspase in the TPI914 N-acyltri-amine positional scanning combinatorial library. The chemical name listed below each box is the reagent from which the R group was derived. Each functional group has the same stereochemistry as the reagent from which it was derived.

Figure 5 shows structures for the individual compounds found to be derepressors of an XIAP-inhibited caspase in the TPI914 N-acyltri-amine library. The chemical name listed at each table entry is the reagent from which the R group was derived. Each functional group has the same stereochemistry as the reagent from which it was derived.

Figure 6 shows structures of the defined functionalities in the mixtures found to be derepressors of an XIAP-inhibited caspase in the TPI927 polyphenylurea positional scanning combinatorial library. The chemical name in each box is the reagent from which the R group was derived. Each functional group has the same stereochemistry as the reagent from which it was derived. For structures 25, 73, 86 and 88, where the core structure of the molecule is modified, the resulting modified core structure and R group is shown.

Figure 7 shows structures of the defined functionalities in the mixtures found to be derepressors of an XIAP-inhibited caspase in the TPI882 C-6-acylamino bicyclic guanidine library. The chemical name in each box is the reagent from which the R group was derived. Each functional group has the same stereochemistry as the reagent from which it was derived.

Figure 8 shows structures of the defined functionalities in the mixtures found to be derepressors of an XIAP-inhibited caspase in the TPI759 N-benzyl-1,4,5-trisubstituted-2,3-diketopiperazine positional scanning  
5 combinatorial library. The chemical name listed below each box is the reagent from which the R group was derived. Each functional group has the same stereochemistry as the reagent from which it was derived.

10 Figure 9 shows structures for individual compounds found to be derepressors of an XIAP-inhibited caspase in the TPI927 polyphenylurea library. The chemical name in each box is the reagent from which the R group was derived. Each functional group has the same  
15 stereochemistry as the reagent from which it was derived.

Figure 10 shows structures for individual compounds found to be derepressors of an XIAP-inhibited caspase in the TPI882 C-6-acylamino bicyclic guanidine  
20 library. The chemical name in each box is the reagent from which the R group was derived. Each functional group has the same stereochemistry as the reagent from which it was derived.

Figure 11 shows dose response of mixtures  
25 identified as derepressors of XIAP-inhibited caspase from the TPI 1239 library. Values shown are for the ratio of  $V_{max}$  for hydrolysis of AC-DEVD-AFC in the presence and absence of each mixture. TPI 1239 mixtures were present at the doses listed at the top of each column.

Figure 12 shows the structures of  
L-3-(2-thienyl)-alanyl-L-(2-naphthyl)-alanyl-  
L-p-chloro-phenylalanyl-  
L-(e-fluorenylmethyloxycarbonyl)-lysine (TPI792-33; Core  
5 peptide 16) and L-3-(2-thienyl)-alanyl,  
L-(2-naphthyl)-alanyl-  
D-(e-fluorenylmethyloxycarbonyl)-lysyl-  
L-(e-fluorenylmethyloxycarbonyl)-lysine (TPI792-35; Core  
peptide 17).

10           Figure 13 shows the effects of VP-16  
(etoposide), TPI792-35, TPI792-33 and the SMAC AVPI  
tetrapeptide (SEQ ID NO:4) on prostate cancer cell  
viability.

Figure 14 shows the generalized structures for  
15 phenyl urea compounds in the TPI 1396 library and  
diketopiperazine compounds in the TPI 1391 library (Panel  
A) and structures for compounds TPI 1391-28, TPI 1391-21,  
TPI 1396-34, TPI 1396-22, TPI 1396-11, TPI 1396-12 (Panel  
B).

20           Figure 15 shows concentration-dependent killing  
of Jurkat leukemia cells by TPI 1391-28 and TPI 1396-34.

Figure 16 shows killing of Jurkat leukemia cells  
by TPI 1391-28 and TPI 1396-34 compared to control  
compounds having similar core pharmacophores,  
25 respectively, but different R groups.

Figure 17 shows a comparison of the effects of  
TPI 1396-34 and TPI 1391-28 on normal bone marrow cells  
versus Jurkat leukemia cells.



Figure 18 shows the effects of over-expression of wild-type XIAP on the apoptogenic activity of TPI 1396-34.

Figure 19 shows the effects of over-expression of wild-type XIAP on the apoptogenic activity of TPI 1396-34.

Figure 20 shows structures for TPI792-3, TPI792-9, TPI792-15, TPI792-17, TPI792-19, TPI792-22, TPI792-27, TPI792-33 and TPI792-35.

Figure 21A shows structures for TPI 1349-1 through TPI 1349-34 along with respective molecular weights, masses and lowest concentration of each agent having a ratio of 1.8 or higher in SMAC competition assays. Figure 21B shows the activity of TPI 1349-1 through TPI 1349-34 in the derepression assay using full length XIAP. Figure 21C shows the activity of TPI 1349-1, -3, -8, -13, -23, and -28 using both full length XIAP and XIAP BIR2 domain. Figure 21D shows the activity of TPI 1349-1, -3, -8, -13, -23, and -28 using cIAP BIR2 domain.

Figure 22A shows structures of TPI 1396-1 through TPI 1396-65 along with respective molecular weights, masses and lowest concentration of each agent having a ratio of 1.8 or higher in SMAC competition assays. Figure 22B shows the activity of TPI 1396-1 through TPI 1396-36 in the derepression assay using full length XIAP. Figure 22C shows the activity of TPI 1396-37 through TPI 1396-65 in the derepression assay using full length XIAP. Figure 22D shows a table indicating the activities of TPI 1396-11, -12, -22, -28, and -34 in the

derepression assay using full length XIAP and the XIAP BIR2 domain. Figure 22E shows the activity of TPI 1396-11, -12, -22, -28, and -34 at 50 µg/ml using XIAP BIR2 domain. Figure 22F shows the activity of TPI 1396-11, -12, -22, -28, and -34 at 100 µg/ml using full length XIAP and Caspase 3 or 7. Figure 22G shows the activity of TPI 1396-11, -12, -22, -28, and -34 at 100 µg/ml using cIAP BIR2 domain.

Figure 23A shows structures of TPI 1391-1 through TPI 1391-36 along with respective molecular weights, masses and lowest concentration of each agent having a ratio of 1.8 or higher in SMAC competition assays. Figure 23B shows the activity of TPI 1391-1 through TPI 1391-36 at 100 µg/ml in the derepression assay using full length XIAP. Figure 23C shows the activity of TPI 1391-1 through TPI 1391-36 at 25 µg/ml in the derepression assay using full length XIAP. Figure 23D shows a table indicating the activities of TPI 1391-1, -4, -5, 7, -17, -21, -25, -28, -34 and -35 in the derepression assay using full length XIAP. Figure 23E shows a comparison of the activities of TPI 1391-1, -4, -5, 7, -17, -21, -25, -28, -34 and -35 in the derepression assay using full length XIAP or XIAP BIR2 domain. Figure 23F shows the activity of TPI 1391-1, -4, -5, 7, -17, -21, -25, -28, -34 and -35 using cIAP BIR2 domain.

Figure 24 shows structures of TPI 1400-1 through TPI 1400-58 along with respective molecular weights, masses and lowest concentration of each agent having a ratio of 1.8 or higher in SMAC competition assays. Figure 24B shows the activity of TPI 1400-1 through TPI 1400-28 at 25 µg/ml in the derepression assay using full length

XIAP. Figure 24C shows the activity of TPI 1400-1 through TPI 1400-28 at 10  $\mu\text{g/ml}$  in the derepression assay using full length XIAP. Figure 24D shows the activity of TPI 1400-29 through TPI 1400-58 at 25  $\mu\text{g/ml}$  in the  
5 derepression assay using full length XIAP. Figure 24E shows the activity of TPI 1400-29 through TPI 1400-58 at 10  $\mu\text{g/ml}$  in the derepression assay using full length XIAP. Figure 24F shows a table indicating the activities of TPI 1400-6, -7, 13, -14, -33, -37, -43, -44 in the  
10 derepression assay using full length XIAP. Figure 24G shows a comparison of the activities of TPI 1400-6, -7, 13, -14, -33, -37, -43, -44 in the derepression assay using full length XIAP or XIAP BIR2 domain. Figure 24H shows the activity of TPI 1400-6, -7, 13, -14, -33, -37, -  
15 43, -44 using cIAP BIR2 domain.

Figure 25a and b show screening of small molecule poly-phenylurea compounds in a Caspase derepression assay to identify compounds that overcome  
20 XIAP-mediated repression of Caspase-3. Figure 25a shows the results using aliquots from the poly-phenylurea library mixtures and Figure 25b shows the results for 36 individual compounds based on deconvolution of the poly-phenylurea library.

25 Figure 26 a, b, c, d, e, and f show characterization of the biochemical mechanism of poly-phenylurea compounds. Figure 26a, b, c, and d show the results of a Caspase derepression assay using poly-phenylurea compounds with XIAP and Caspase-3 (a), XIAP and  
30 Caspase-9 (b), BIR-2 and Caspase-3 (c) and p35 and Caspase-3 (d). Figure 26e shows a binding assay where biotinylated SMAC (7-mer) was adsorbed to Neutravidin-

coated plates then GST-XIAP was added with or without compounds. Bound GST-XIAP was detected with an anti-GST antibody. Figure 26f shows a binding assay where GST-XIAP was adsorbed to plates and then incubated with  
5 biotinylated-SMAC (7-mer) with or without compounds. Bound biotinylated-SMAC peptide was detected by a streptavidin-europium-based fluorescence method.

Figure 27 a, b, c, d, e, and f show characterization of cellular activity of poly-phenylurea  
10 compounds. Figure 27a and b show cell death after incubation of Jurkat leukemia cells with various poly-phenylurea compounds (a) or TPI 1396-34 (b). Figure 27c shows Caspase3/7 activity after incubation of Jurkat cells with various compounds. Figure 27d shows cell death in  
15 Jurkat cells cultured with various concentrations of TPI 1396-34 with or without zVAD-fmk. Figure 27e shows cell death after incubation of U937 cells that stably over-express XIAP or neomycin with TPI 1396-34. Inset shows immunoblot analysis of lysates prepared from the U937  
20 cells. Figure 27f shows cell death of HeLa cells transfected with XIAP, Bcl-XL or CrmA and incubated with TPI 1396-34. Figure 27g shows viability of control and SV40-transfected cells treated with TPI 1396-34.

Figure 28 a, b, c and d show broad anti-tumor  
25 activity of TPI 1396-34. Figure 28a shows the result of cell growth of sixty human tumor cell lines cultured with TPI 1396-34 or TPI 1396-28 compared to cells treated with solvent alone. Each line represents a tumor cell line. Figure 28b shows the effect of TPI 1396-34 on normal  
30 versus malignant cells. Figure 28c shows the mean (+/- standard deviation) percent apoptosis of CLL B-cells from

five patients cultured with various poly-phenylurea compounds. Figure 28d shows cell death of AML cells isolated from 5 patients and cultured with various concentrations of TPI 1396-34. All samples were treated  
5 with active TPI 1396-34 and inactive TPI 1396-28 as well as AVPI peptide, but the complete data set is shown only for AML-1. Comparable results were obtained with the other samples.

Figure 29 shows the effects of TPI 1396-12, TPI  
10 1396-22, and TPI 1396-11 on 60 tumor cell lines from the NCI-60 cell panel on cell growth compared to cells cultured with solvent diluent alone.

Figure 30 a and b show TPI 1396-34 sensitizes cancer cells to chemotherapy and TRAIL. Figure 30a shows  
15 viability of DU145 prostate cancer cells cultured with Etoposide (VP16), Doxorubicin (DOX) or Paclitaxel (Tax) with or without TPI 1396-34. Figure 30b shows viability of cancer cell lines treated with various concentrations of TRAIL alone or in combination with TPI 1396-34.

20 Figure 31 a, b, c and d show the effect of combination of conventional chemotherapeutic agents with TPI 1396-34 on various tumor cell lines. The viability of DU145 (a), PPC1 (b), PC3 (c), and H460 (d) cells cultured with various concentrations of TPI 1396-34 and various  
25 concentrations of chemotherapeutic drugs is shown.

Figure 32 a, b and c show anti-tumor activity of TPI 1396-34 in clonogenic survival assays and tumor xenograft studies. Figure 32a shows colony number of two prostate cancer cell lines, PC-3 and LNCaP, cultured with

TPI 1396-34. Control compound is represented by the bars, showing only the 10  $\mu$ M dose results. Figure 32b shows tumor size in Balb/C *nu-/nu-* mice injected with PPC1 prostate cancer cells after treatment with TPI 1396-34.

5 The inset shows tumor weight in mice sacrificed at 24 days after compound injections. Figure 32c shows tumor volume and tumor weight in Balb/C *nu-/nu-* mice injected with HCT116 colon cancer cells. On days 6, 7, and 8 mice were treated with TPI 1396-34 (I) or solvent control (C) and  
10 tumor volume was measured. On day 19, the mice were sacrificed and the tumors were weighed. Bars represent the median tumor size or weight.

Figure 33 a and b show anti-tumor activity of poly-phenylurea compounds in a tumor xenograft model.

15 Figure 33a shows tumor volume of Balb/C *nu-/nu-* mice injected with PPC-1 prostate cancer cells treated with TPI 1396-22, TPI 1396-34 or solvent control. Figure 33b shows tumor weight of mice sacrificed on day 19. Bars represent the median tumor size or weight.

20 Figure 34 shows structures of agents TPI 1509-1 through TPI 1509-9 along with respective molecular weights, masses and lowest concentration of each agent having a ratio of 1.8.

Figures 35 A and B show compound modifications  
25 of R groups based on TPI 1509-7 as an example of SAR studies for poly-phenylurea compounds.

Figure 36A shows structures of TPI 1332-1 through TPI 1332-93 along with respective molecular weights. Figure 36B shows structures of selected TPI 1332

compounds, including TPI 1332-4, TPI 1332-24, TPI 1332-41, TPI 1332-69, TPI 1332-76, and TPI 1332-77 along with respective molecular weights and the activity of the compounds in competing with XIAP BIR2 domain binding to the SMAC peptide. Figure 36C shows the activity of 1332-1 through TPI 1332-93 at 50 µg/ml in the derepression assay using full length XIAP. Figure 36D shows the activity of TPI 1332-1 through TPI 1332-93 at 16.7 µg/ml in the derepression assay using full length XIAP. Figure 36E shows the activity of TPI 1332-1, -4, -41, -53, -69, and 77 in the derepression assay using full length XIAP and the XIAP BIR2 domain. Figure 36F shows the activity of TPI 1332-1, -2, -4, -6, -41, -47, -53, -55, -69, -76, -77 and -85 using cIAP BIR2 domain.

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Figure 37 shows that none of TPI 1495-1, -2, -3, -4, -6, -7, -8 or -9 compete tetrapeptide series compete with XIAP binding to the SMAC peptide, while TPI 1495-5 does compete tetrapeptide series compete with XIAP binding to the SMAC peptide; also shown is that TPI 1495-2, -3, -4, -6, -7, and -8 are inactive in the derepression assay using full length XIAP, while TPI 1495-1, -5, and -9 are active in the derepression assay.

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Figure 38 shows that TPI 1396-34 functions by targeting XIAP protein. Cells from XIAP knock-out mice or wild type mice were treated with either TPI 1396-34 or daunorubicin and % viability was assessed. Cells used in these studies were either untransformed (Figure 38 A and C) or transformed with a retrovirus encoding SC40 large T antigen (Figure 38 B and D).

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Figure 39 shows that TPI 1396-34 enhances cytotoxicity of antigen-specific CTL. Tumor cells treated with specific antigen were incubated with antigen-specific T cells at an effector:target ratio of either 5 (Figure 39B) or 10 (Figure 39A). Percent cell lysis as a function of antigen concentration is shown.

Figure 40 shows that TPI 1396-12 effects *in vivo* activation of caspases. Figure 40A shows an immunoblot of tumor tissue from animals treated with control or TPI 1396-12, performed using antibodies specific for cleaved caspase-3 or actin. Figure 40B shows immunohistochemistry of tumor tissues from animals treated with control or TPI 1396-12, performed using hematoxylin and eosin (nuclear stain) (A and B); caspase-3 antibodies and PCNA antibodies (C and D); caspase-6 antibodies (E and F) and DFF 40 antibodies (G and H).

Figure 41 shows toxicological analysis of TPI 1396-12, including white blood cell count (A); red blood cell count (B); platelet count (C); BUN (D); Bilirubin (E); ALT (F); and AST (G).

Figure 42 shows that both TPI 1540-14 (a) and TPI 1540-15 (b) selectively binds to the BIR2 domain of XIAP, while inactive compound TPI 1540-20 does not (c).

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Figure 43 shows the activity of TPI 1453-1 (also referred to as TPI 792-33 or TPI 1408-3), TPI 1453-2, TPI 1453-3, TPI 1453-4, TPI 1453-5, TPI 1453-6 (also referred to as TPI 792-35), TPI 1453-7, TPI 1453-8, and TPI 1453-9, in the derepression assay using full length XIAP (XIAP-FL

30



derepression data), as well as in the SMAC competition assay (Competitive binding assay).

Figure 44 shows a table of biotinylated tetrapeptides of the TPI 1554 series, as well as the corresponding peptide number for the original tetrapeptides (Non-biotin Synthesis #) and molecular weights (MW).

Figures 45A-J show binding of BID and XIAP-BIR2 to biotinylated peptides as follows: (A) TPI 1453-1 (TPI 1554-1); (B) TPI 1453-6 (TPI 1554-2); (C) TPI 1332-4 (TPI 1554-3); (D) TPI 1332-41 (TPI 1554-4); (E) TPI 1332-69 (TPI 1554-5); (F) TPI 1332-77 (TPI 1554-6); (G) TPI 1495-19 (TPI 1554-7); (H) TPI 1495-20 (TPI 1554-8); (I) SMAC 7-mer (TPI 1465-1, -2); and (J) SMAC 4-mer (TPI 1465-3, -4).

Figures 46 A-C show binding of three concentrations of XIAP-BIR2 to biotinylated tetrapeptides, with Figure 46A showing results using 1  $\mu\text{g/ml}$  XIAP BIR2; Figure 46B showing results using 0.5  $\mu\text{g/ml}$  XIAP BIR2, and Figure 46C showing results using 0.25  $\mu\text{g/ml}$  XIAP BIR2.

Figure 47 shows competition of the binding of biotinylated tetrapeptides with XIAP-BIR2, using (A) biotinylated TPI 1332-69, which is a non-SMAC mimic; and (B) biotinylated TPI 1332-4, which is a SMAC mimic.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides agents that suppress an inhibitor of apoptosis protein (IAP) from

inhibiting the protease activity of a caspase or from binding to a caspase. An advantage of an agent of the invention is that it can be used to allow apoptosis to occur in a cell where apoptosis is being prevented by the regulatory activity of an IAP. Accordingly, the invention provides methods for reducing the ability of a population of cells to survive *in vitro* or *ex vivo* by administering to the cells an agent that derepresses an IAP-inhibited caspase. Use of an agent having specificity for a particular IAP-inhibited caspase in such a method can selectively target and kill a sub-population of cells in a larger mixed population. Also provided is a method of treating an individual having a condition characterized by a pathologically reduced level of apoptosis, such as cancer or hyperplasia, by administering to the individual an agent of the invention, wherein the agent derepresses an IAP inhibited caspase, thereby increasing the level of apoptosis.

The invention further provides methods for identifying agents that modulate inhibitors of apoptosis. Using the methods of the invention a candidate agent can be tested for the ability to suppress an inhibitor of apoptosis (IAP) protein from inhibiting a protease activity of a caspase or from binding to a caspase. A caspase when uninhibited mediates apoptosis. Thus, an agent determined by the methods to derepress an IAP-inhibited caspase is identified as an agent that allows apoptosis to occur in the presence of negative regulatory components. An advantage of the methods of the invention is that they can be performed in a high throughput format such that large libraries of candidate agents can be

efficiently screened for identification of a variety of derepressors of an IAP-inhibited caspase.

As used herein the term "caspase" is intended to mean a member of the family of cysteine aspartyl-specific proteases that cleave C-terminal to an aspartic acid residue in a polypeptide and are involved in cell death pathways leading to apoptosis. The term is intended to be consistent with its use in the art as described, for example, in Martin and Green, Cell 82:349-352 (1995). The caspases previously were referred to as the "Ice" proteases, based on their homology to the first identified member of the family, the interleukin-1 $\beta$  (IL-1 $\beta$ ) converting enzyme (Ice), which converts the inactive 33 kiloDalton (kDa) form of IL-1 $\beta$  to the active 17.5 kDa form. The Ice protease was found to be homologous to the *Caenorhabditis elegans* ced-3 gene, which is involved in apoptosis during *C. elegans* development, and transfection experiments showed that expression of Ice in fibroblasts induced apoptosis in the cells (see Martin and Green, *supra*, 1995). Therefore, the term includes Ice and ced-3.

Additional polypeptides sharing homology with Ice and ced-3 have been identified and are referred to as caspases, each caspase being distinguished by a number. For example, the originally identified Ice protease now is referred to as caspase-1, the protease referred to as caspase-3 previously was known variously as CPP32, YAMA and apopain, and the protease now designated caspase-9 previously was known as Mch6 or ICE-LAP6. The caspase family of proteases are characterized in that each is a cysteine protease that cleaves C-terminal to an aspartic acid residue and each has a conserved active site cysteine

comprising generally the amino acid sequence QACXG (SEQ ID NO:1), where X can be any amino acid and often is arginine. The caspases are further subcategorized into those that have DEVD (SEQ ID NO:2) cleaving activity, including caspase-3 and caspase-7, and those that have YVAD (SEQ ID NO:3) cleaving activity, including caspase-1 (Martin and Green, *supra*, 1995).

As used herein the term "IAP" or "inhibitor of apoptosis" is intended to mean a protein that inhibits the proteolytic activity of a caspase. The term can include a protein that when bound to a caspase inhibits the proteolytic activity of the caspase. The term can also include a protein that inhibits the proteolytic activity of a downstream caspase by inhibiting the ability of an upstream caspase to process a precursor of the caspase to a mature form. Also included in the term is a protein that induces ubiquitination and degradation of a caspase.

Members of the Inhibitor of Apoptosis (IAP) protein family of antiapoptotic proteins are conserved across evolution with homologues found in both vertebrate and invertebrate animal species. The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be identified based on their ability to functionally complement defects in the cell death inhibitor p35, a baculovirus protein that binds to and inhibits caspase. Subsequently, at least seven additional human homologues have been identified and demonstrated to inhibit cell death including X chromosome linked IAP (XIAP, Genbank accession number U32974); cellular IAP proteins, c-IAP-1/HIAP-2/hMIHB and c-IAP-2/ HIAP-1/hMIHC (Liston et al., Nature 379:349-353 (1996); Rothe et al., Cell 83:1243-1252

(1995)); neuronal apoptosis inhibitory protein, NAIP (Roy et al., Cell 80:167-178 (1995)); ML-IAP also referred to as LIVIN (Vucic et al., Cur. Biol. 10:1359-1366 (2000) and Kasof et al., J. Biol. Chem. 276:3238-3246 (2001));

5 Apollon (Chen et al., Biochem. Biophys. Res. Commun. 264:847-854 (1999)); and survivin (Ambrosini et al., Nature Med. 3:917-921 (1997)). Two Drosophila homologues (DIAP1 and DIAP2) have also been identified and demonstrated to inhibit cell death (Deveraux et al., Genes

10 and Development 13:239-252 (1999)). A central role for IAP-family proteins in programmed cell death regulation in Drosophila has been suggested by the finding that several apoptosis-inducing proteins in flies, including reaper, hid, and grim bind to IAPs as part of their cytotoxic

15 mechanism. Other IAP proteins include viral IAPs such as CiIAP, PoIAP, CpIAP and ASF IAP (Deveraux et al., *supra* (1999)).

IAP proteins targeted by an agent of the invention include those that inhibit the activity of an

20 effector caspase such as caspase-3 or caspase-7 and those that inhibit an initiator caspase such as caspase-9. The human IAPs (XIAP, cIAP1, and cIAP2) have been reported to bind and potently inhibit caspase-3 and -7, with  $K_i$ s in the range of 0.2-10 nM. These caspases operate in the

25 distal portions of apoptotic protease cascades, functioning as effectors rather than initiators of apoptosis.

A common structural feature of all IAP family members is a ~70 amino acid motif termed baculoviral IAP

30 repeat (BIR), which is present in one to three copies as described, for example, in Deveraux et al., Genes and

Development 13:239-252 (1999). The conserved presence and spacing of cysteine and histidine residues observed within BIR domains indicates that the structure represents a zinc binding domain. BIR domains have been shown to exhibit  
 5 distinct functions. For example, the second BIR domain of XIAP (BIR2) is a potent inhibitor for caspase-3, whereas the third BIR domain of XIAP (BIR3) targets caspase-9 (see Wu et al., Nature 408:1008-1012 (2000)). In addition to the BIR motif located at the N-terminal and central  
 10 portions of IAP, a RING finger domain is located in the C-terminal portion of members of the IAP protein family (Birnbaum et al., J. Virol. 68:2521-2528 (1994)). A BIR domain corresponds to an amino acid sequence having the consensus sequence: Xaa1-Xaa1-Xaa1-Arg-Xaa3-Xaa1-Xaa4-Xaa5-Xaa1-Xaa1-Trp-Xaa6-Xaa1-Xaa1-Xaa2-Xaa1-Xaa3-Xaa1-Xaa1-Xaa1-Xaa1-Leu-Ala-Xaa1-Ala-Gly-Phe-Xaa3-Xaa3-Xaa1-Gly-Xaa1-Xaa1-Asp-Xaa1-Val-Xaa1-Cys-Phe-Xaa1-Cys-Xaa1-Xaa1-Xaa1-Xaa3-Xaa1-Xaa1-Trp-Xaa1-Xaa1-Xaa1-Xaa7-Xaa1-Xaa1-Xaa1-Xaa1-His-Xaa1-Xaa8-Xaa1-Xaa1-Pro-Xaa1-Cys-  
 15 Xaa1-Xaa5-Xaa3 (SEQ ID NO: 16), wherein Xaa1 is any amino acid, Xaa2 is any amino acid or is absent, Xaa3 is a hydrophobic amino acid (for example, Ala, Cys, Ile, Leu, Met, Phe, Pro, Trp, Tyr, or Val), Xaa4 is serine or threonine, Xaa5 is phenylalanine or tyrosine, Xaa6 is  
 20 proline or is absent, Xaa7 is aspartic or glutamic acid, and Xaa8 is a basic amino acid (for example, Arg, His, or Lys).

As used herein the term "IAP-inhibited caspase"  
 30 is intended to mean a cysteine aspartyl-specific protease that is prevented or suppressed from proteolytic activity due to the presence of an inhibitor of apoptosis protein. The term can include a cysteine aspartyl-specific protease

having reduced activity due to a bound inhibitor of apoptosis protein. The term can also include a cysteine aspartyl-specific protease that is prevented or suppressed from being processed to a mature form capable of  
5 proteolytic activity due to the presence of an inhibitor of apoptosis protein. An example of a non-processed cysteine aspartyl-specific protease that is useful in the invention is a pro-caspase having an attached pro-domain. Alternatively, the compositions and methods of the  
10 invention can be directed to an IAP-inhibited caspase that does not contain a prodomain or is not a procaspase.

As used herein the term "derepress," when used in reference to an IAP-inhibited caspase, is intended to mean reduction, inhibition or prevention of the ability of  
15 the IAP to inhibit the proteolytic activity of the caspase. Accordingly, a derepressor of a IAP-inhibited caspase is a molecule that inhibits or prevents the ability of the IAP to inhibit caspase proteolytic activity. The term can include inhibition or prevention  
20 of the ability of an IAP to induce ubiquitination and degradation of caspases.

As used herein, the term "agent" means a synthetic or isolated biological molecule such as a simple or complex organic molecule, a peptide, a peptidomimetic,  
25 a protein or an oligonucleotide that is capable of derepressing an IAP-inhibited caspase.

As used herein, the term "pharmaceutically acceptable carrier" is intended to mean a medium having sufficient purity and quality for use in humans. Such a  
30 medium can be a human pharmaceutical grade, sterile

medium, such as water, sodium phosphate buffer, phosphate buffered saline, normal saline or Ringer's solution or other physiologically buffered saline, or other solvent or vehicle such as a glycol, glycerol, an oil such as olive  
5 oil or an injectable organic ester. Pharmaceutically acceptable media are substantially free from contaminating particles and organisms.

As used herein the term "inhibiting," when used in reference to a protein activity, is intended to mean a  
10 reduction in the activity by decreasing affinity of the protein for a substrate or decreasing the catalytic rate at which the protein converts a substrate to product. The term includes, for example, decreasing the affinity of an IAP for a caspase substrate, decreasing the affinity of a  
15 caspase for a polypeptide substrate, decreasing the rate at which a caspase cleaves a polypeptide C-terminal to an aspartic acid residue, or decreasing the rate at which a caspase is ubiquitinated or proteolytically degraded.

As used herein the term "isolated," when used in  
20 reference to an agent, means that the agent is separated from 1 or more reagent, precursor or other reaction product. Therefore, an isolated agent is an agent that is free from one or more compounds found in the synthetic reaction or reaction pathway that produces the agent.  
25 Also included in the term is an agent that is free from one or more compound that it is found with in nature. An isolated agent also includes a substantially pure agent. The term can include a molecule that has been produced by a combinatorial chemistry method and separated from  
30 precursors and other products by chemical purification or by binding to second molecule with sufficient stability to



be co-purified with the second molecule. The term can include naturally occurring agents such as products of biosynthetic reactions or non-naturally occurring agents.

As used herein the term "peptide" refers to a molecule containing two or more amino acids linked by a covalent bond between the carboxyl of one amino acid and the amino group of another. Invention peptides can be included in larger molecules or agents, such as larger peptides, proteins, fragments of proteins, peptoids, peptidomimetics and the like. A peptide can be a non-naturally occurring molecule, which does not occur in nature, but is produced as a result of *in vitro* methods, or can be a naturally occurring molecule such as a protein or fragment thereof expressed from a cDNA library. Peptides can be either linear, cyclic or multivalent, and the like, which conformations can be achieved using methods well-known in the art. The term includes molecules having naturally occurring proteogenic amino acids as well as non-naturally occurring amino acids such as D-amino acids and amino acid analogs, any of which can be incorporated into a peptide using methods known in the art. In view of this definition, one skilled in the art would know that reference herein to an amino acid, unless specifically indicated otherwise, includes, for example, naturally occurring proteogenic L-amino acids, D-amino acids, chemically modified amino acids such as amino acid analogs, naturally occurring non-proteogenic amino acids such as norleucine, and chemically synthesized agents. Exemplary amino acids useful in the invention are described further below.

As used herein, the term "proteogenic," when used in reference to an amino acid, indicates that the amino acid can be incorporated into a protein in a cell through well known metabolic pathways. The amino acids are designated as D or L in reference to the configuration at the alpha carbon. Amino acids referred to herein without specific reference to configuration are understood to have the L configuration at the alpha carbon. Proteogenic amino acids are indicated herein using the single letter or three letter code and are intended to be consistent with the nomenclature used in the art as described for Example in Branden and Tooze Introduction to Protein Structure, Garland Publishing, New York, pp6-7 (1991). Other amino acids are indicated using nomenclature known in the art, wherein, for example, pClPhe refers to p-chloro-phenylalanine, ThiAla refers to 2-thienyl-alanine, Nal refers to 3-(2-naphthyl)-alanine, 3I-Tyr refers to 3-iodo-Tyrosine, Cha refers to cyclohexylalanine, Lys-e-Fmoc refers to lysine(e-fluorenylmethoxycarbonyl) and OEt-Tyr refers to Tyrosine(O-ethyl).

As used herein the term "core" is intended to mean a chemical structure or motif of a molecule, or portion thereof. The chemical structure or motif can be, for example, an amino acid sequence of a peptide or peptide containing molecule, or a chemical formula representing the covalent attachment of atoms in a molecule. A chemical structure or motif included in the term can be further defined with respect to chirality. A core peptide or other chemical entity need not be located at the center of a molecule.

The present invention provides isolated agents that derepresses an IAP-inhibited caspase. An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

5 (L-Ala)-X<sub>1</sub>-(L-Trp)-X<sub>2</sub> (Core peptide 4)

where X<sub>1</sub> is L-Trp or D-Trp and X<sub>2</sub> is L-ThiAla or L-pClPhe. Exemplary core peptides included in Core peptide 4 include, for example:

10 (L-Ala)-(L-Trp)-(L-Trp)-(L-ThiAla) (Core peptide 5),

(L-Ala)-(L-Trp)-(L-Trp)-(L-pClPhe) (Core peptide 6) and

15 (L-Ala)-(D-Trp)-(L-Trp)-(L-ThiAla) (Core peptide 7).

An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub> (Core peptide 23)

20 where X<sub>1</sub> is L-Ala, L-Cha, L-Nal, D-Trp or D-Trp(CHO); X<sub>2</sub> is D-Nal, D-Trp, D-Trp(CHO), L-Trp, L-Trp(CHO), D-Cha or D-ThiAla; X<sub>3</sub> is L-Trp, L-Trp(CHO) or D-Phe; and X<sub>4</sub> is L-Nal, D-Nal, D-Trp, D-Trp(CHO), L-ThiAla, L-3I-Tyr or L-pClPhe. Exemplary core peptides included in Core peptide 25 23 include, for example:

- (L-Ala) - (D-Nal) - (L-Trp) - (L-Nal) (Core peptide  
24)
- (D-Trp) - (D-Trp) - (L-Trp) - (D-Nal) (Core peptide  
25)
- 5 (L-Cha) - (D-Nal) - (L-Trp) - (L-ThiAla) (Core peptide  
26)
- (L-Ala) - (L-Trp) - (L-Trp) - (L-3I-Tyr) (Core peptide  
27)
- 10 (L-Ala) - (D-Trp) - (L-Trp) - (L-ThiAla) (Core peptide  
28)
- (L-Cha) - (L-Trp) - (L-Trp) - (L-pClPhe) (Core peptide  
29)
- (L-Ala) - (D-Trp) - (L-Trp) - (D-Trp) (Core peptide  
30)
- 15 (L-Ala) - (D-Trp) - (D-Phe) - (D-Trp) (Core peptide  
31)
- (L-Nal) - (D-Trp) - (D-Phe) - (D-Trp) (Core peptide  
32)
- 20 (L-Nal) - (D-Cha) - (L-Trp) - (D-Trp) (Core peptide  
33)
- (L-Nal) - (D-ThiAla) - (D-Phe) - (D-Trp) (Core peptide  
34) .

An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

$X_1-X_2-X_3-X_4$  (Core peptide 8)

- 5 where  $X_1$  is D-Nal or L-ThiAla;  $X_2$  is Lys- $\epsilon$ Fmoc, D-pClPhe or L-Nal;  $X_3$  is D-Nal, L-pClPhe or D-Lys(Fm); and  $X_4$  is Lys- $\epsilon$ Fmoc or D-pFPhe. Exemplary core peptides included in Core peptide 8 include, for example:

10 (D-Nal)-(Lys- $\epsilon$ Fmoc)-(L-pClPhe)-(Lys- $\epsilon$ Fmoc)  
(Core peptide 9)

(D-Nal)-(D-pClPhe)-(L-pClPhe)-(Lys- $\epsilon$ Fmoc) (Core peptide 10)

(D-Nal)-(L-Nal)-(L-pClPhe)-(Lys- $\epsilon$ Fmoc) (Core peptide 11)

15 (D-Nal)-(L-Nal)-(D-Lys- $\epsilon$ Fmoc)-(Lys- $\epsilon$ Fmoc) (Core peptide 12)

(L-ThiAla)-(Lys- $\epsilon$ Fmoc)-(D-Nal)-(Lys- $\epsilon$ Fmoc)  
(Core peptide 13)

20 (L-ThiAla)-(Lys- $\epsilon$ Fmoc)-(L-pClPhe)-(pF-D-F) (Core peptide 14)

(L-ThiAla)-(D-pClPhe)-(L-pClPhe)-(Lys- $\epsilon$ Fmoc)  
(Core peptide 15)

(L-ThiAla)-(L-Nal)-(L-pClPhe)-(Lys-εFmoc) (Core peptide 16)

(L-ThiAla)-(L-Nal)-(D-Lys-εFmoc)-(Lys-εFmoc)  
(Core peptide 17).

- 5           An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

$X_1$ - $X_2$ - $X_3$ - $X_4$  (Core peptide 35)

- where  $X_1$  is L-ThiAla or Phe;  $X_2$  is D-pClPhe or D-OEt-Tyr ;  
10  $X_3$  is D-Nal, or D-OEt-Tyr; and  $X_4$  is D-pClPhe or D-pNO<sub>2</sub>Phe.  
Exemplary core peptides included in Core peptide 35 include, for example:

(L-ThiAla)-(D-pClPhe)-(D-Nal)-(D-pClPhe) (Core peptide 36)

- 15           (L-ThiAla)-(D-pClPhe)-(D-Nal)-(D-pNO<sub>2</sub>Phe) (Core peptide 37)

(L-ThiAla)-(D-OEt-Tyr)-(D-OEt-Tyr)-(D-pClPhe)  
(Core peptide 38)

- 20           (Phe)-(D-pClPhe)-(D-Nal)-(D-pClPhe) (Core peptide 39).

An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

A-X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub> (Core peptide 18)

where X<sub>1</sub> is Met, Ser, Thr, Trp, or ThiAla and X<sub>2</sub> and X<sub>3</sub> are selected from Ala, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Tyr, D-Ala, D-Asp, D-Glu, D-Phe, D-His, D-Ile, D-Lys, D-Leu, D-Met, D-Asn, D-Pro, D-Gln, D-Arg, D-Ser, D-Thr, D-Val, D-Trp, D-Tyr, L-Nle, D-Nle, L-Cha, D-Cha, L-PyrAla, D-PyrAla, L-ThiAla, D-ThiAla, L-Tic, D-Tic, L-pClPhe, D-pClPhe, L-pIPhe, D-pIPhe, L-pNO<sub>2</sub>Phe, D-pNO<sub>2</sub>Phe, L-Nal, D-Nal, beta-Ala, e-Aminocaproic acid, L-Met[O<sub>2</sub>], L-dehydPro, or L-3I-Tyr.

An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

X<sub>1</sub>-X<sub>2</sub>-(L-Trp)-(D-Trp) (Core peptide 19)

where X<sub>1</sub> and X<sub>2</sub> are selected from Ala, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Tyr, D-Ala, D-Asp, D-Glu, D-Phe, D-His, D-Ile, D-Lys, D-Leu, D-Met, D-Asn, D-Pro, D-Gln, D-Arg, D-Ser, D-Thr, D-Val, D-Trp, D-Tyr, L-Nle, D-Nle, L-Cha, D-Cha, L-PyrAla, D-PyrAla, L-ThiAla, D-ThiAla, L-Tic, D-Tic, L-pClPhe, D-pClPhe, L-pIPhe, D-pIPhe, L-pNO<sub>2</sub>Phe, D-pNO<sub>2</sub>Phe, L-Nal, D-Nal, beta-Ala, e-Aminocaproic acid, L-Met[O<sub>2</sub>], L-dehydPro, or L-3I-Tyr.

An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to:

$X_1-X_2-X_3-X_4-W-W$  (Core peptide 55),

where  $X_1$ ,  $X_2$  and  $X_3$  are selected from Ala, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Cys or Tyr and  $X_4$  is selected from Ala,  
 5 His, Lys, Asn, Gln, Arg, Ser, Thr or Val.

An agent that derepresses an IAP-inhibited caspase can have a core peptide or amino acid sequence motif corresponding to any of:

$X_1-X_2-A-A-W-W$  (Core peptide 43), SEQ ID NO: 7

10  $X_1-X_2-G-A-W-W$  (Core peptide 44), SEQ ID NO: 8

$X_1-X_2-R-A-W-W$  (Core peptide 45), SEQ ID NO: 9

$X_1-X_2-X_4-A-W-W$  (Core peptide 46),

$X_1-X_2-C-K-W-W$  (Core peptide 47), SEQ ID NO: 10

$X_1-X_2-L-X_3-W-W$  (Core peptide 20),

15

$X_1-X_2-R-X_3-W-W$  (Core peptide 21),

$X_1-X_2-G-X_3-W-W$  (Core peptide 22),

20

$X_1-X_2-T-X_3-W-W$  (Core peptide 42),

$X_1-X_2-V-X_3-W-W$  (Core peptide 48),

$X_1-T-X_2-X_3-W-W$  (Core peptide 49),



$X_1$ -Y- $X_2$ - $X_3$ -W-W (Core peptide 50),

A- $X_1$ - $X_2$ - $X_3$ -W-W (Core peptide 51),

C- $X_1$ - $X_2$ - $X_3$ -W-W (Core peptide 52),

F- $X_1$ - $X_2$ - $X_3$ -W-W (Core peptide 53), or

5           K- $X_1$ - $X_2$ - $X_3$ -W-W (Core peptide 54),

where  $X_1$ ,  $X_2$  and  $X_4$  are selected from Ala, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Cys or Tyr and  $X_3$  is selected from Ala, Lys, Ser or Thr.

10           The core peptide sequences of the invention can be those of a molecule or a portion of a molecule. For example, the above-described sequences having four positions can be tetrapeptide molecules and the above-described sequences having four or six positions can be  
15 hexapeptide molecules. A core peptide of the invention can also be included in larger molecules including, for example, a molecule having at least 5 amino acids, at least 6 amino acids, at least 7 amino acids, at least 8 amino acids, at least 9 amino acids, at least 10 amino  
20 acids, at least 20 amino acids or at least 25 amino acids. In some embodiments, the amino acid lengths of molecules comprising invention peptides can be defined by a maximum length including, for example, no more than about 4, no more than about 5, no more than about 6, no more than  
25 about 7, no more than about 8, no more than about 9, no more than about 10, no more than about 20, no more than about 25, no more than about 50, no more than about 100,

no more than about 150, or no more than about 200 or more amino acids in length so long as the peptide is capable of derepressing an IAP-inhibited caspase. A molecule having a core peptide of the invention can also be defined within  
5 a size range delimited by a combination of any of the above described minimum and maximum lengths.

The invention further provides agents that are effective derepressors of an IAP-inhibited caspase having non-peptide based core structures. Thus, the invention  
10 provides an agent that derepresses an IAP-inhibited caspase and having a core structure corresponding to an N-benzyl-1,4,5-trisubstituted-2,3-diketopiperazine such as TPI 759 shown in Figure 8. An agent having the TPI 759 core structure can be substituted, for example, at  
15 position R1 derived from an amino acid side chain group of norleucine, NapAla, cyclohexylalanine, Lys, norvaline or valine; at R2 derived from an amino acid side chain group of Leu, NapAla, Phe, Ile or Val; and at R3 with the functional group derived from 4-isobutyl-alpha-  
20 methylphenylacetic acid, 3,5-bis(trifluoromethyl)-phenylacetic acid, heptanoic acid, (alpha-alpha-alpha-trifluoro-m-tolyl)acetic acid, 4-tert-butyl-cyclohexane carboxylic acid, m-tolylacetic acid, 3,4-dichlorophenylacetic acid, 3,3-diphenyl propionic acid,  
25 dicyclohexylacetic acid, cycloheptanecarboxylic acid, p-Tolylacetic acid or cyclohexanebutyric acid as shown in Figure 8.

An agent that derepresses an IAP-inhibited caspase can have a core structure corresponding to a C-6-  
30 acylamino bicyclic guanidine such as TPI 882 shown in Figure 7. An agent having the TPI 882 core structure can

be substituted, for example, at position R1 derived from an amino acid side chain group of L-cyclohexylalanine, D-cyclohexylalanine, D-2-chloroPhe, O-ethyl-D-Tyr, p-iodo-L-Phe, p-iodo-D-Phe, D-homo-Phe, L-homo-Phe, L-naphthylAla, 5 D-naphthylAla or L-4,4-biphenylalanine; at position R2 with the functional group derived from 2-phenylbutyric acid, 3-phenylbutyric acid, m-tolylacetic acid, 3-fluorophenylacetic acid, p-tolylacetic acid, 4-fluorophenylacetic acid, 3-methoxyphenylacetic acid, 4-10 methoxyphenylacetic acid, 4-ethoxyphenylacetic acid, 4-biphenylacetic acid, phenylacetic acid, 4-phenylbutyric acid, heptanoic acid, 4-methylvaleric acid, tert-butyric acid, cyclohexylcarboxylic acid, cyclohexylacetic acid, cyclohexylbutyric acid, cycloheptanecarboxylic acid, 15 cyclobutanecarboxylic acid, cyclopentylcarboxylic acid, 3-cyclopentylpropionic acid, cyclohexylpropionic acid, 4-methyl-1-cyclohexylcarboxylic acid, 4-t-butylcyclohexylcarboxylic acid, 2-norbornaneacetic acid, 1-adamantane acetic acid, 2-ethylbutyric acid, 3,3-20 diphenylpropionic acid or cyclopentylacetic acid; and at position R3 with the functional group derived from 3-fluorophenylacetic acid, 4-ethoxyphenylacetic acid, 4-biphenylacetic acid or 3,5-bis(trifluoromethyl)phenylacetic acid as shown in Figure 25 7. An agent having the TPI 882 core structure can be substituted at R2 and R3 with the functional group derived from phenylacetic acid and at R1 derived from an amino acid side chain group of L-cyclohexylalanine, D-cyclohexylalanine, D-p-chloro-Phe, D-p-fluoro-Phe, L-p-30 fluoro-Phe, D-2-chloro-Phe, O-ethyl-L-Tyr, O-ethyl-D-Tyr, O-methyl-D-Tyr, 3,5-diiodo-Tyr or L-naphthylAla; at R1 with an amino acid side chain group of Phe; at R3 with the functional group derived from phenylacetic acid and at R2

with the functional group derived from p-tolylacetic acid, 4-fluorophenylacetic acid, 3-methoxyphenylacetic acid, 4-methoxyphenylacetic acid, 4-ethoxyphenylacetic acid, 4-biphenylacetic acid, phenylacetic acid, 4-phenylbutyric  
 5 acid, heptanoic acid, 3-methylvaleric acid or 4-methylvaleric acid; or at R1 with an amino acid side chain group of Phe; at R2 with the functional group derived from phenylacetic acid; and at R3 with the functional group derived from 4-biphenylacetic acid, cyclohexanecarboxylic  
 10 acid, cyclohexylacetic acid, cyclohexylbutyric acid, cycloheptanecarboxylic acid, 3-cyclopentylpropionic acid or 3,5-bis(trifluoromethyl)phenylacetic acid as shown in Figure 10.

An agent that derepresses an IAP-inhibited  
 15 caspase can have a core structure corresponding to a polyphenylurea such as TPI 927 shown in Figure 6. An agent having the TPI 927 core structure can be substituted, for example, at position R1 derived from an amino acid side chain group of D-Lys(Me), L-3-(2Nap)Ala,  
 20 D-Chala, L-Phe, Pro, Leu or Ser; at position R2 derived from an amino acid side chain group of  $\epsilon$ -Lys, L-Nle, D-Phe, Pro, D-Orn(Me), Gln, L-3-(2-Nap)Ala or D-Thr; and at position R3 with the functional group derived from 4-methoxyphenylacetic acid, 1-adamantaneacetic acid,  
 25 cyclohexanebutyric acid, 4-tert-butylcyclohexanecarboxylic acid, cycloheptanecarboxylic acid, 3-fluorophenylacetic acid, 3,3-diphenylpropionic acid, 4-ethoxyphenylacetic acid, 1-phenyl-1-cyclopropanecarboxylic acid, 1-naphthylacetic acid, or cyclobutane carboxylic acid as  
 30 shown in Figure 6. An agent having the TPI 927 core structure can be substituted at R1 and R2 with an amino acid side chain group of Phe and at R3 with the functional

group derived from trimethylacetic acid, hydrocinnamic acid, 4-tert-butylcyclohexane carboxylic acid, 4-methyl-1-cyclohexanecarboxylic acid, cyclopentylacetic acid, 1-phenyl-1-cyclopropanecarboxylic acid,

5 cyclohexanecarboxylic acid, phenylacetic acid, cycloheptanecarboxylic acid, cyclobutane carboxylic acid, cyclohexanebutyric acid, 1-adamantaneacetic acid, cyclopentanecarboxylic acid, isobutyric acid, cyclohexylacetic acid; 3-methoxyphenylacetic acid, butyric

10 acid, 3-(3,4,5)-trimethoxyphenylpropionic acid; heptanoic acid; 2-norbornaneacetic acid, cyclohexanepropionic acid, tert-butyric acid, 4-ethoxyphenylacetic acid, 3,3-diphenylpropionic acid, 4-methoxyphenylacetic acid, acetic acid, methylvaleric acid p-tolylacetic acid or 4-isobutyl-

15 alpha-methylphenylacetic acid as shown in Figure 9.

An agent that derepresses an IAP-inhibited caspase can have a core structure corresponding to an N-acyltriamine such as TPI 914 shown in Figure 4. An agent having the TPI 914 core structure can be substituted, for

20 example, at position R1 derived from an amino acid side chain group of Nap-Ala or 4-Fluoro-phenylalanine; at position R2 derived from an amino acid side chain group of L-Trp, Nap-Ala, D-Trp, 4-chlorophenylalanine, D-cyclohexylalanine or Tyr; and at R3 with the functional

25 group derived from 4-vinylbenzoic acid, 4-ethyl-4-biphenylcarboxylic acid, 3,5-Bis(trifluoromethyl)-phenylacetic acid, 4-biphenylcarboxylic acid, 4-biphenylacetic acid or 3,5-bis-(trifluoromethyl)-benzoic acid as shown in Figure 4. An agent having the TPI 914

30 core structure can be substituted at R1 with a functional group derived from an amino acid side chain group of Leu, at R2 with a functional group derived from an amino acid

side chain group of D-Trp and at R3 with methyl; at R1  
with a functional group derived from an amino acid side  
chain group of Leu, at R2 with a functional group derived  
from an amino acid side chain group of Phe and at R3 with  
5 the functional group derived from 3,5-  
Bis(trifluoromethyl)-phenylacetic acid; at R1 with a  
functional group derived from an amino acid side chain  
group of Leu, at R2 with a functional group derived from  
an amino acid side chain group of Phe and at R3 with the  
10 functional group derived from 4-vinylbenzoic acid; or at  
R1 with a functional group derived from an amino acid side  
chain group of Leu, at R2 with a functional group derived  
from an amino acid side chain group of Phe and at R3 with  
the functional group derived from 4-ethyl-4-  
15 biphenylcarboxylic acid each as shown in Figure 5.

Those skilled in the art will recognize that  
libraries having the core structure of TPI 914, TPI 927,  
TPI 759, TPI 882, can be combinatorialized at one or more  
position. A combinatorialized position refers to a  
20 position which is variously substituted with different  
moieties such that a library of molecules  
combinatorialized at the position is a mixture of  
molecules that differ in chemical structure at that  
position. Such libraries can be used to identify agents  
25 that derepress an IAP-inhibited caspase, for example, in a  
screen utilizing positional scanning as described in  
Example VI. Thus, any one of positions R1, R2 or R3 can  
be held fixed to a discrete moiety while the remaining two  
positions are combinatorialized, thereby generating  
30 sublibraries based on which position is fixed. Moreover,  
one can add additional positions to the core structure  
that can be combinatorialized or held constant while one

or more other positions are combinatorialized. Thus, different or more diverse libraries can be created based on a particular core structure or on a species identified from the library as capable of derepressing an IAP-  
5 inhibited caspase.

Those skilled in the art will understand that an agent of the invention having a core structure corresponding to TPI 914, TPI 927, TPI 759, TPI 882, such as a compound of the TPI 1396, TPI 1349, TPI 1391 or TPI  
10 1400 series, can further include one or more attached moieties such as a peptide moiety. An agent of the invention can be multivalent, as described above, in which case the attached moiety can be one or more core structures corresponding to TPI 914, TPI 927, TPI 759, TPI  
15 882, a core peptide having a sequence described above; or a combination of one or more of these core structures and core peptides.

An agent that is capable of derepressing an IAP-inhibited caspase, whether based on a peptide or non-  
20 peptide core structure can include a moiety known to naturally occur in biological proteins. Such moieties when part of a protein are commonly referred to as amino acid R-groups. These R groups can be characterized by a variety of physical or chemical properties. Taking the  
25 essential amino acids as an example, the R groups found on Gly, Ala, Val, Leu, or Ile have the characteristic of being non-polar; polar R groups include the sulfhydryl moiety of Cys, the thioether of Met, hydroxyl moieties of Ser and Thr, and amide moieties of Asn and Gln; Asp and  
30 Glu are characterized as polar acidic groups due to the presence of carboxylic acid moieties; polar basic R groups

include Lys which has an amino moiety, Arg which has a guanidino moiety and His which has an imidazole with secondary amines; and Phe, Trp, Tyr, and His are characterized as aromatic amino acids due to the presence  
5 of phenyl or heterocyclic rings. An agent of the invention can include one or more of these moieties or characteristics, thereby rendering the agent capable of derepressing an IAP-inhibited caspase.

An agent of the invention can also be described  
10 or characterized according to other moieties or combinations of moieties that when present renders the agent capable of derepressing an IAP-inhibited caspase. Definitions for various moieties that can be present in the agents of the invention are set forth below.

15

As used herein, the term "alkyl," alone or in combination, refers to a saturated, straight-chain or branched-chain hydrocarbon moiety containing from 1 to 10, preferably from 1 to 6 and more preferably from 1 to 4,  
20 carbon atoms. Examples of such moieties include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl, decyl and the like.

The term "alkene," alone or in combination,  
25 refers to a straight-chain or branched-chain hydrocarbon moiety having at least one carbon-carbon double bond in a total of from 2 to 10, preferably from 2 to 6 and more preferably from 2 to 4, carbon atoms. Examples of such moieties include, but are not limited to, ethenyl, E- and  
30 Z-propenyl, isopropenyl, E- and Z-butenyl, E- and Z-isobutenyl, E- and Z-pentenyl, decenyl, methylenidene



(=CH<sub>2</sub>), ethylidene (-CH=CH-), propylidene (-CH<sub>2</sub>-CH=CH-) and the like.

The term "alkyne," alone or in combination, refers to a straight-chain or branched-chain hydrocarbon moiety having at least one carbon-carbon triple bond in a total of from 2 to 10, preferably from 2 to 6 and more preferably from 2 to 4, carbon atoms. Examples of such moieties include, but are not limited to, ethynyl (acetylenyl), propynyl (propargyl), butynyl, hexynyl, decynyl and the like.

The term "cycloalkyl," alone or in combination, refers to a saturated, cyclic arrangement of carbon atoms which number from 3 to 8 and preferably from 3 to 6, carbon atoms. Examples of such cycloalkyl moieties include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl and the like.

The term "aryl" refers to a carbocyclic (consisting entirely of carbon and hydrogen) aromatic group selected from the group consisting of phenyl, naphthyl, indenyl, indanyl, azulenyl, fluorenyl, and anthracenyl; or a heterocyclic aromatic group selected from the group consisting of furyl, thienyl, pyridyl, pyrrolyl, oxazolyly, thiazolyl, imidazolyl, pyrazolyl, 2-pyrazolinyl, pyrazolidinyl, isoxazolyl, isothiazolyl, 1,2,3-oxadiazolyl, 1,2,3-triazolyl, 1,3,4-thiadiazolyl, pyridazinyl, pyrimidinyl, pyrazinyl, 1,3,5-triazinyl, 1,3,5-trithianyl, indoliziny, indolyl, isoindolyl, 3H-indolyl, indolinyl, benzo[b]furanyl, 2,3-dihydrobenzofuranyl, benzo[b]thiophenyl, 1H-indazolyl, benzimidazolyl, benzthiazolyl, purinyl, 4H-quinoliziny,

quinolinyl, isoquinolinyl, cinnolinyl, phthalazinyl, quinazolinyl, quinoxalinyl, 1,8-naphthyridinyl, pteridinyl, carbazolyl, acridinyl, phenazinyl, phenothiazinyl, and phenoxazinyl.

5 "Aryl" groups, as defined in this application may independently contain one to four substituents which are independently selected from the group consisting of hydrogen, halogen, hydroxyl, amino, nitro, trifluoromethyl, trifluoromethoxy, alkyl, alkenyl,  
 10 alkynyl, cyano, carboxy, carboalkoxy, 1,2-dioxyethylene, alkoxy, alkenoxy or alkynoxy, alkylamino, alkenylamino, alkynylamino, aliphatic or aromatic acyl, alkoxy-carbonylamino, alkylsulfonylamino, morpholinocarbonylamino, thiomorpholinocarbonylamino, N-  
 15 alkyl guanidino, aralkylaminosulfonyl; aralkoxyalkyl; N-aralkoxyurea; N-hydroxylurea; N-alkenylurea; N,N-(alkyl, hydroxyl)urea; heterocyclyl; thioaryloxy-substituted aryl; N,N-(aryl, alkyl)hydrazino; Ar'-substituted sulfonylheterocyclyl; aralkyl-substituted heterocyclyl;  
 20 cycloalkyl and cycloalkenyl-substituted heterocyclyl; cycloalkyl-fused aryl; aryloxy-substituted alkyl; heterocyclylamino; aliphatic or aromatic acylaminocarbonyl; aliphatic or aromatic acyl-substituted alkenyl; Ar'-substituted aminocarbonyloxy; Ar', Ar'-  
 25 disubstituted aryl; aliphatic or aromatic acyl-substituted acyl; cycloalkylcarbonylalkyl; cycloalkyl-substituted amino; aryloxycarbonylalkyl; phosphorodiamidyl acid or ester;

"Ar'" is a carbocyclic or heterocyclic aryl  
 30 group as defined above having one to three substituents selected from the group consisting of hydrogen, halogen,

hydroxyl, amino, nitro, trifluoromethyl, trifluoromethoxy, alkyl, alkenyl, alkynyl, 1,2-dioxymethylene, 1,2-dioxyethylene, alkoxy, alkenoxy, alkynoxy, alkylamino, alkenylamino or alkynylamino, alkylcarbonyloxy, aliphatic  
5 or aromatic acyl, alkylcarbonylamino, alkoxy carbonylamino, alkylsulfonylamino, N-alkyl or N,N-dialkyl urea.

The term "alkoxy," alone or in combination, refers to an alkyl ether moiety, wherein the term "alkyl" is as defined above. Examples of suitable alkyl ether  
10 moieties include, but are not limited to, methoxy, ethoxy, n-propoxy, iso-propoxy, n-butoxy, iso-butoxy, sec-butoxy, tert-butoxy and the like.

The term "alkenoxo," alone or in combination, refers to a moiety of formula alkenyl-O-, wherein the term  
15 "alkenyl" is as defined above. Examples of suitable alkenoxy moieties include, but are not limited to, allyloxy, E- and Z-3-methyl-2-propenoxy and the like.

The term "thioalkoxy" refers to a thioether moiety of formula alkyl-S-, wherein alkyl is as defined  
20 above.

The term "alkylamino," alone or in combination, refers to a mono- or di-alkyl-substituted amino group (i.e., a group of formula alkyl-NH- or (alkyl)<sub>2</sub>-N-), wherein the term "alkyl" is as defined above. Examples of  
25 suitable alkylamino moieties include, but are not limited to, methylamino, ethylamino, propylamino, isopropylamino, t-butylamino, N,N-diethylamino and the like.

The term "amide" refers to either  $-N(R^1)-C(=O)-$  or  $-C(=O)-N(R^1)-$  where  $(R^1)$  is defined herein to include hydrogen as well as other groups. The term "substituted amide" refers to the situation where  $(R^1)$  is not hydrogen, while the term "unsubstituted amide" refers to the situation where  $(R^1)$  is hydrogen.

The term "aryloxy," alone or in combination, refers to a moiety of formula aryl-O-, wherein aryl is as defined above. Examples of aryloxy moieties include, but are not limited to, phenoxy, naphthoxy, pyridyloxy and the like.

The term "arylamino," alone or in combination, refers to a moiety of formula aryl-NH-, wherein aryl is as defined above. Examples of arylamino moieties include, but are not limited to, phenylamino (anilido), naphthylamino, 2-, 3- and 4-pyridylamino and the like.

The term "aryl-fused cycloalkyl," alone or in combination, refers to a cycloalkyl moiety which shares two adjacent atoms with an aryl moiety, wherein the terms "cycloalkyl" and "aryl" are as defined above. An example of an aryl-fused cycloalkyl moiety is a benzofused cyclobutyl group.

The term "alkylcarbonylamino," alone or in combination, refers to a moiety of formula alkyl-CONH, wherein the term "alkyl" is as defined above.

The term "alkoxycarbonylamino," alone or in combination, refers to a moiety of formula  $\text{alkyl-OCONH-}$ , wherein the term "alkyl" is as defined above.

The term "alkylsulfonylamino," alone or in  
5 combination, refers to a moiety of formula  $\text{alkyl-SO}_2\text{NH-}$ , wherein the term "alkyl" is as defined above.

The term "arylsulfonylamino," alone or in combination, refers to a moiety of formula  $\text{aryl-SO}_2\text{NH-}$ , wherein the term "aryl" is as defined above.

10 The term "N-alkylurea," alone or in combination, refers to a moiety of formula  $\text{alkyl-NH-CO-NH-}$ , wherein the term "alkyl" is as defined above.

The term "N-arylurea," alone or in combination, refers to a moiety of formula  $\text{aryl-NH-CO-NH-}$ , wherein the  
15 term "aryl" is as defined above.

The term "halogen" means fluorine, chlorine, bromine and iodine.

In view of the above definitions, other chemical terms used throughout this application can be easily  
20 understood by those of skill in the art. Terms may be used alone or combined to describe a combination of moieties according to accepted chemical nomenclature.

An agent of the invention can be synthesized using reagents and conditions well known to yield products  
25 having predictable moieties or characteristics. For example, peptides can be synthesized in large numbers at

relatively low cost and they can be readily modified to exhibit diverse properties (see, for example, Rees et al., Protein Engineering: A Practical Approach (IRL Press 1992)). A peptide derepressor of an IAP-inhibited caspase  
5 can be synthesized using a modification of the solid phase peptide synthesis method (Merrifield (J. Am. Chem. Soc., 85:2149 (1964); Houghten, U.S. Patent No. 4,631,211, issued Dec. 23, 1986) or can be synthesized using standard solution methods well known in the art (see, for example,  
10 Bodanszky, M., Principles of Peptide Synthesis 2nd ed. (Springer-Verlag, 1988 and 1993, suppl.)). Peptides prepared by the method of Merrifield can be synthesized using an automated peptide synthesizer such as the Applied Biosystems 431A-01 Peptide Synthesizer (Mountain View, CA)  
15 or using a manual peptide synthesis method (Houghten, *supra*, 1986).

Furthermore, combinatorial methods such as those described below can be used to make an agent that derepresses an IAP-inhibited caspase. A library can be  
20 synthesized to have candidate agents with particular moieties such as those defined above or described in the Examples set forth below. Additionally, the synthetic conditions can be selected to produce a library of candidate compounds with particular characteristics  
25 inherent in one or more of the moieties described herein such as the characteristics described above for amino acid R groups. For example, a library can be synthesized to have characteristics of SMAC a naturally occurring IAP inhibitor. The N-terminal region of SMAC has been shown  
30 to mediate binding to and inhibition of IAPs (see Srinivasula et al., Nature 410:112-116 (2001), Wu et al., Nature 408:1008-1012 (2000), Liu et al., Nature 408:1004-

1008 (2000)). Accordingly, this N-terminal domain can be used to guide library synthesis such that reactants and conditions are chosen to selectively incorporate similar moieties and characteristics into the candidate agents in the library. Similar strategies can be employed using agents described herein or identified by the methods of the invention, wherein a library is made to selectively contain characteristics or moieties found in a particular agent or common to a plurality of agents. Such a design strategy increases the probability that an effective derepressor of an IAP-inhibited caspase will be identified.

An agent of the invention that is capable of derepressing an IAP-inhibited caspase can be identified in a screen or otherwise characterized according to any of a variety of functional properties described herein. In one embodiment a derepressor of an IAP-inhibited caspase is identified or otherwise characterized based on its ability to allow caspase activity in the presence of an IAP. For example, the effectiveness of a compound of the invention can be determined according to the ratio of caspase activity for an IAP-inhibited caspase in the presence and absence of an agent of the invention.

Using caspase derepression assays, several compounds have been disclosed herein that derepress an IAP-inhibited caspase. For example, the invention provides an isolated agent having a core structure selected from any of the structures shown in Figures 21-24, where the agent is selected from TPI 1349-1 through 1349-34; TPI 1396-1 through TPI 1396-36; TPI 1391-1

through TPI 1391-36; and TPI 1400-1 through TPI 1400-58 and where the agent derepresses an IAP-inhibited caspase.

A compound of the invention that derepress an IAP-inhibited caspase can be a member of a disclosed compound class, such as a polyphenylurea, diketopiperazine, bicyclic guanidine, N-acyl triamine, or a tetrapeptide. A summary of various activities observed for compound classes disclosed herein is presented in Table XII, below. This table shows average activities of representative compounds from the polyphenylurea, diketopiperazine, bicyclic guanidine, N-acyl triamine, and tetrapeptide classes in the caspase derepression assay, SMAC competition assay and the Jurkat cell cytotoxicity assay. Polyphenylurea and diketopiperazines were found to have activity in the enzyme derepression assay in the presence of either full length XIAP or XIAP BIR2 domain, as is described in Example VIII.

Table XII

IAP Antagonists			
<i>Families of Compounds</i>			
Compound Class	Enzyme Derepress IC-50 ( $\mu$ M)	SMAC competition	Cell Activity IC-50 ( $\mu$ M)
Poly-phenylurease	12	No	7
Diketopiperazines	32	No	8
Bicyclic guanidines	32	N.T.	N.T.
N-Acyl triamines	53	N.T.	N.T.
Tetrapeptides-1	19	No	8
Tetrapeptides-2	9	Yes	40



An exemplary assay for identifying a compound that derepresses an IAP-inhibited caspase is provided in Example I and use of the assay to identify such derepressor compounds is demonstrated in Examples I through VI. As described below in the Examples, a ratio of  $V_{\max}$  in the presence and absence of the agent for an IAP-inhibited caspase that is at least about 1.7, depending upon assay conditions, is indicative of an effective derepressor of an IAP-inhibited caspase. Those skilled in the art will understand that a value for this ratio that is indicative of effectiveness will depend upon the concentration of the agent used and the  $IC_{50}$  of the agent. Accordingly, when higher concentrations of the agent are used the threshold value for the ratio of  $V_{\max}$  in the presence and absence of the agent can be at least about 2 at least about 2.5, at least about 3 or at least about 4 or higher. When lower concentrations of the agent are used this ratio can be as low as at least about 1.5, at least about 1.3 at least about 1 or lower. Thus, it can be appropriate to express the ratio in combination with the relative amount of agent to IAP present in the assay including, for example, 1 molar equivalent of agent per IAP, 2 molar equivalents of agent per IAP, 5 molar equivalents of agent per IAP, 10 molar equivalents of agent per IAP or 50 molar equivalents of agent per IAP or higher.

An agent that derepresses an IAP-inhibited caspase can also be identified by its affinity for an IAP or a caspase-binding fragment thereof, for example, in a binding assay. It will be understood that a functional fragment of an IAP, caspase or both can be used in a binding assay to identify a derepressor of an

IAP-inhibited caspase. Affinity of an agent for an IAP determined using a binding assay can, if desired, be quantified by an equilibrium dissociation constant ( $K_d$ ) or equilibrium association constant ( $K_a$ ). An agent that  
5 derepresses an IAP-inhibited caspase can be identified as an agent that has a  $K_d$  that is in the micromolar range including, for example, less than about  $1 \times 10^{-6}$  M,  $1 \times 10^{-7}$  M, or  $1 \times 10^{-8}$  M. Higher affinity agents can also be identified including an agent having nanomolar range  
10 affinity such as a  $K_d$  less than about  $1 \times 10^{-9}$  M,  $1 \times 10^{-10}$  M or  $1 \times 10^{-11}$  M. An agent of the invention can also have picomolar affinity including, for example, a  $K_d$  that is less than  $1 \times 10^{-12}$  M.

Alternatively, the effectiveness of an agent at  
15 derepressing an IAP-inhibited caspase can be determined based on inhibition of the association between an IAP and caspase, for example, in an inhibition binding assay. It will be understood that a functional fragment of an IAP, caspase or both can be used in an inhibition binding  
20 assay. Alternatively, a derepressor of an IAP-inhibited caspase can be identified based on its ability to inhibit binding between IAP and another inhibitor such as SMAC. An exemplary assay for determining inhibition of IAP binding to SMAC is provided in Example VII. Inhibition  
25 can be quantified, if desired, by an equilibrium inhibition constant, such as  $K_i$ . Values for  $K_i$  can be determined by performing derepression assays, such as those described herein, with increasing concentrations of the agent and a fixed concentration of each binding  
30 partner. Binding or inhibition can be analyzed to determine the equilibrium constants described above using well known kinetic analysis such as those described in

Segel, Enzyme Kinetics John Wiley and Sons, New York (1975). An agent that derepresses an IAP-inhibited caspase can be identified as those having  $K_i$  in the micromolar, nanomolar or picomolar ranges such as those  
5 ranges and values described above for  $K_d$ .

Accordingly, the invention provides a complex having an IAP bound to an agent, the agent having a core peptide or core structure of the invention including, for example, those core structures described above. The  
10 complex can be isolated from at least one other cellular component normally occurring with the IAP in nature. For example, the complex can be in a purified state being substantially free of other cellular components that normally occur with the IAP in nature. The complex can  
15 also occur in a recombinant cell that does not normally express the IAP.

The invention further provides conjugates including a moiety linked to an agent that derepresses an IAP-inhibited caspase. A conjugate of the invention can  
20 include a moiety useful for targeting the agent to a particular cell or for increasing the stability or biological half life of the agent that derepresses an IAP-inhibited caspase. For example, a moiety can be a particular antibody, functional fragment thereof, or other  
25 binding polypeptide that has specificity for a particular cell in which it is desired to promote apoptosis, such as a tumor cell. Any moiety capable of targeting the agent to a cell in which an IAP-inhibited caspase is to be derepressed can be used as a conjugate.

A conjugate of an agent that derepresses an IAP-inhibited caspase can also be a moiety capable of introducing the agent to the cytosol of a cell or otherwise facilitating passage of the agent through the cell membrane. An agent can be introduced into the cell by, for example, a heterologous targeting domain or using a lipid based carrier. Thus, the invention provides cytosolic delivery of an agent that derepresses an IAP-inhibited caspase.

A moiety can also be a drug delivery vehicle such as a chambered microdevice, a cell, a liposome or a virus that provides stability or properties otherwise advantageous for administration of the agent that derepresses an IAP-inhibited caspase. Generally, such microdevices, should be nontoxic and, if desired, biodegradable. Various moieties, including microcapsules, which can contain an agent, and methods for linking a moiety, including a chambered microdevice, to a therapeutic agent are well known in the art and commercially available (see, for example, "Remington's Pharmaceutical Sciences" 18th ed. (Mack Publishing Co. 1990), chapters 89-91; Harlow and Lane, Antibodies: A laboratory manual (Cold Spring Harbor Laboratory Press 1988); see, also, Hermanson, *supra*, 1996).

In addition, a derepressor of an IAP-inhibited caspase formulation can be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the agent is released systemically over time. Osmotic minipumps also can be used to provide

controlled delivery of specific concentrations of the derepressor of an IAP-inhibited caspase species and formulations through cannulae to the site of interest, such as directly into a tumor growth or into the vascular supply of a tumor. The biodegradable polymers and their use are described, for example, in detail in Brem et al., J. Neurosurg. 74:441-446 (1991).

A conjugate of the invention can include a moiety that is a label. A labeled agent that binds to an IAP and/or caspase can be used to identify the subcellular localization of the IAP and/or caspase or to identify a previously unidentified IAP or caspase. A labeled agent that binds to an IAP and/or caspase can also be used to identify other molecules that interact with an IAP and/or caspase. As described in further detail below, such a binding competition assay can be used to identify an agent that derepresses an IAP-inhibited caspase. A label that can be incorporated as a moiety includes, for example, a fluorophore, chromophore, paramagnetic spin label, radionucleotide, or binding group having specificity for another molecule that can be detected.

A labeled agent of the invention can be useful for identifying cells within a tissue that are inhibited from apoptosis by an IAP-inhibited caspase. Thus, the labeled agent can be used in a diagnostic method to identify cells for which administration of a derepressor of an IAP-inhibited caspase will allow apoptosis to proceed. The method can include steps of administering a labeled agent of the invention to a tissue and identifying one or more cells that incorporate the labeled agent. The labeled agent can be administered using methods for in

vivo delivery as described above. The diagnostic methods can be used at a variety of resolutions. For example, the method can be carried out to identify a tissue containing cells labeled by the agent. Alternatively, higher  
5 resolution methods can be used to identify a particular cell or cell type within a tissue that is labeled in the presence of an IAP-inhibited caspase. Because the diagnostic methods can be used to distinguish a cell for which administration of a derepressor of an IAP-inhibited  
10 caspase will allow apoptosis to proceed from non-labeled cells, the methods can be useful for guiding in the choice of targeting or delivery conjugate to use in a therapeutic method of the invention.

The diagnostic method can be performed *in vitro*  
15 in which case the labeled agent can be administered by injection or by soaking the tissue in a solution containing the labeled agent. Again the methods can be used at a resolution sufficient to distinguish within a tissue a cell having an IAP-inhibited caspase over those  
20 that are not inhibited from apoptosis in this way. Such resolution can be achieved for example, by use of a microscopic based technique. Further resolution can provide subcellular localization of an IAP-inhibited caspase. Subcellular localization can be used to  
25 determine an appropriate cytosolic delivery conjugate or to further identify the role of apoptosis in the particular tissue or cells under study.

The invention also provides a pharmaceutical composition containing a derepressor of an IAP-inhibited  
30 caspase and a pharmaceutical carrier. Such compositions can be used in the apoptosis promoting methods of the

invention to inhibit, treat or reduce the severity of a pathological condition characterized by a pathologically reduced level of apoptosis. For example, a derepressor of an IAP-inhibited caspase can be administered as a solution or suspension together with a pharmaceutically acceptable medium.

The derepressor of an IAP-inhibited caspase formulations include those applicable for parenteral administration such as subcutaneous, intraperitoneal, intramuscular, intravenous, intradermal, intracranial, intratracheal, and epidural administration. As well as formulations applicable for oral, rectal, ophthalmic (including intravitreal or intracameral), nasal, topical (including buccal and sublingual), intrauterine, or vaginal administration. The derepressor of an IAP-inhibited caspase formulation can be presented in unit dosage form and can be prepared by pharmaceutical techniques well known to those skilled in the art. Such techniques include the step of bringing into association the active ingredient and a pharmaceutical carrier or excipient.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions such as the pharmaceutically acceptable media described above. The solutions can additionally contain, for example, anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Other formulations include, for example, aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. The formulations can be presented

in unit-dose or multi-dose containers, for example, sealed ampules and vials, and can be stored in a lyophilized condition requiring, for example, the addition of the sterile liquid carrier, immediately prior to use.

- 5 Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules and tablets of the kind previously described.

A pharmaceutically acceptable medium can additionally contain physiologically acceptable compounds  
10 that act, for example, to stabilize the derepressor of an IAP-inhibited caspase agent. Such physiologically acceptable compounds include, for example, carbohydrates such as glucose, sucrose or dextrans; antioxidants such as ascorbic acid or glutathione; chelating agents such as  
15 EDTA, which disrupts microbial membranes; divalent metal ions such as calcium or magnesium; low molecular weight proteins; lipids or liposomes; or other stabilizers or excipients. As described previously, derepressor of an IAP-inhibited caspase formulation also can be formulated  
20 with a pharmaceutically acceptable medium such as a biodegradable polymer. All of the above-described pharmaceutical carriers and media can be what is termed in the art pharmaceutical grade which means that they are of sufficient purity and quality for use in humans and are  
25 distinguishable from comparable reagents in research grade formulations.

The invention also provides a composition including a derepressor of an IAP-inhibited caspase and a molecule having therapeutic activity. A molecule included  
30 with a derepressor of the invention can be a compound having activity against a condition characterized by a



pathologically reduced level of apoptosis. For example, the compound can have activity against cancer. An exemplary compound that has activity against prostate cancer and that can be used in combination with a

5 derepressor compound of the invention is VP-16 (etoposide). As demonstrated by the results of Example X, administration of VP-16 with either TPI 792-33 or TPI 792-35 had a more potent effect on killing cancer cells than any of these compounds alone.

10 Other anti-cancer drugs can also be used in a composition with a derepressor of an IAP-inhibited caspase including, but not limited to, an alkylating agent such as mechlorethamine, chlorambucil, cyclophosphamide, melphalan, ifosfamide; an antimetabolite such as  
15 methotrexate, 6-mercaptopurine, 5-fluorouracil or cytarabine; an antibody such as Rituxan, Herceptin, or MabThera; a plant alkaloid such as vinblastine or vincristine, or etoposide; an antibiotic such as doxorubicin, daunomycin, bleomycin, or mitomycin; a  
20 nitrosurea such as carmustine or lomustine; an inorganic ion such as cisplatin; a biological response modifier such as interferon; an enzyme such as asparaginase; or a hormone such as tamoxifen or flutamide. These and other anti-cancer compounds, including those described herein  
25 below with respect to practicing a therapeutic method of the invention in combination with another therapeutic method, are known in the art and formulations suitable for pharmaceutical use are known as described, for example, in The Merck Manual 16<sup>th</sup> Ed., Merck Res. Labs., Rahway N.J.  
30 (1992). In addition, for treating a condition characterized by a pathologically reduced level of apoptosis, a compound of the invention can be administered

in conjunction with a therapeutic antibody. Such a therapeutic antibody can be, for example, an antibody that modulates apoptosis, such as by binding to an apoptosis regulatory molecule and modulating its activity. As a  
5 non-limiting example, a compound of the invention can be administered in conjunction with an antibody that activates caspase 3, caspase 7, Trail-R1 or Trail R-2. Exemplary Trail-R1 and Trail-R2 monoclonal antibodies are available from Human Genome Sciences, Rockville, MD.

10

The invention provides compounds that demonstrate broad anti-cancer activity alone or in combination with known anti-cancer agents. For example, polyphenylurea compound of the invention such as TPI 1396-  
15 34, TPI 1396-12, TPI 1396-22, and TPI 1396-11 significantly reduce tumor cell growth of sixty different tumor cell lines (see Example XIII and Figures 28 and 29). The concentration of polyphenylurea compound required to kill 50% of the cells (LD50) was comparable or better than  
20 that of known anti-cancer drugs. Toxicological analysis of mice treated with TPI 1396-12 at dosages effective to inhibit tumor growth indicated no toxic effects on a variety of parameters including white blood cell count, red blood cell count, platelet count, BUN, bilirubin, ALT  
25 and AST (see Figure 41 and Example XXIII). In addition, as shown herein, normal cells were relatively resistant to polyphenylurea compounds compared to tumor cell lines (see Figure 28). Polyphenylurea compounds also were demonstrated to induce apoptosis in non-replicating  
30 malignant cells such as chronic lymphocytic leukemia (CLL) and acute myelogenous leukemia (AML) cells isolated from patients (see Figure 28). Additional studies revealed that a polyphenylurea compound of the invention can

enhance cytotoxicity of antigen-specific CTL (see Figure 39).

As further disclosed herein, polyphenylurea compounds can collaborate with conventional anticancer  
5 drugs to induce killing of tumor cells. For example, TPI 1396-34 significantly increases dose-dependent cytotoxicity of etoposide (VP16), doxorubicin (DOX) or paclitaxel (TAXOL) in various cancer cell lines (see Example XIV and Figures 30 and 31). Similar effects on the induction of  
10 apoptosis were seen using polyphenylurea compounds and the biological agent TRAIL, which is an apoptosis inducing member of the Tumor Necrosis Factor (TNF) family (see Figure 30).

The invention also provides compounds that  
15 demonstrate anti-tumor activity in clonogenic survival assays and *in vivo*. For example, the polyphenylurea compound TPI 1396-34 decreased clonogenic survival of various cancer cell lines in a concentration dependent manner (see Example XV and Figure 32). In addition, as  
20 disclosed herein, polyphenylurea compounds such as TPI 1396-34 and TPI 1396-22 have anti-tumor activity *in vivo*. For example, these compounds significantly reduced tumor size and tumor weight in human tumor xenografts grown in immunocompromised mice (see Example XV and Figure 32 and  
25 33). Additional studies confirmed that the polyphenylurea compounds of the invention function *in vivo* by modulating caspase activity (see Figure 40), and that XIAP protein is indeed the target of these compounds *in vivo* (see Figure 38).

30 The invention further provides a kit, including at least one compound of the invention that has activity

as a derepressor of an IAP-inhibited caspase and a second compound having therapeutic activity. A compound of the invention that can be included in a kit includes, for example, a compound having a core peptide selected from  
5 the group consisting of Core peptides 4 through 39 and 42 through 55, or having a core structure selected from any of the structures shown in Figures 5, 9, 10, 12, 14B, 21-24, 34, 35, 36, 37 and 43, wherein the compound derepresses an IAP-inhibited caspase. Such kits are  
10 useful, for example, in the treatment of a condition characterized by a pathologically reduced level of apoptosis. For example, a kit including VP-16 with either TPI 792-33 or TPI 792-35 can be used to treat prostate cancer.

15           A suitable kit includes compounds as separately packaged formulations or in a mixed formulation, so long as the compounds are provided in an amount sufficient to have a therapeutic effect following at least one administration of each compound. The formulations can be  
20 any of those described above, or otherwise known to be appropriate for the particular compound and mode of administration.

          The contents of a kit of the invention are housed in packaging material or other suitable physical  
25 structure, preferably to provide a sterile, contaminant-free environment. In addition, the packaging material contains instructions indicating how the materials within the kit can be administered for treatment of a condition characterized by a pathologically reduced level of  
30 apoptosis. The instructions for use typically include a tangible expression describing the route of administration

or, if required, methods for preparing the formulation for administration. The instructions can also include identification of potential effects from use of the kit's contents or a warning regarding improper use of the  
5 contents of the kit.

The invention provides a method of identifying an agent that derepresses an IAP-inhibited caspase. The method includes the steps of (a) contacting an IAP and a caspase with an agent suspected of being able to derepress  
10 an IAP-inhibited caspase, wherein the caspase is an IAP-inhibited caspase that is inhibited by the IAP, wherein the contacting occurs under conditions that allow caspase activity in the absence of the IAP; and (b) detecting derepression of the IAP-inhibited caspase.

15 Derepression of the IAP-inhibited caspase can be detected as an increase in an IAP-inhibited caspase activity including, for example, proteolytic activity. Proteolytic activity can be measured in an *in vitro* assay using a specific substrate. For example, a continuous  
20 fluorometric assay can be used to measure hydrolysis rates by following release of either 7-amino-4-trifluoromethylcoumarin (AFC) from DEVD (SEQ ID NO:2) that is derivatized with a C-terminal aminomethylcoumarin, YVAD (SEQ ID NO:3) that is derivatized with a C-terminal aminomethylcoumarin  
25 (Tyr-Val-Ala-Asp-aminomethylcoumarin), or carbobenzoxy-Glu-Val-Asp-aminomethylcoumarin; or by following the release of p-nitroanilide (pNA) from similar peptides labeled with pNA, as described in U.S. Pat No. 6,228,603 B1.

An immunoblot or other chromatography based assay can be used to detect proteolysis of a substrate by caspase according to altered molecular weight of the products compared to the substrate. For example, the proteolytic activity of an upstream initiator caspase, such as caspase-9, can be determined based on processing of a downstream effector pro-caspase, such as pro-caspase-3, to the mature form in an immunoblot assay as described in U.S. Pat No. 6,228,603 B1. Comparison of the results of such an assay for an IAP-inhibited caspase in the presence and absence of an agent of the invention can be used to identify a derepressor of the IAP-inhibited caspase according to a relative increase in caspase activity in the presence of the agent.

Proteolytic activity of a caspase can also be determined by identifying morphological changes in a cell or a cell nucleus characteristic of apoptosis. Such changes that are characteristic of apoptosis include, for example, chromatin condensation, nuclear fragmentation, cell shrinkage, or cell blebbing leading to the eventual breakage into small membrane surrounded fragments termed apoptotic bodies. Thus, an agent that is a derepressor of an IAP-inhibited caspase can be identified according to the ability to cause a characteristic apoptotic change when added to a cell that is prevented from undergoing apoptosis by an IAP-inhibited caspase. A similar assay can be performed on a cell free extract derived from such a cell so long as an apoptotic change such as chromatin condensation or nuclear fragmentation can be distinguished in the presence and absence of the added agent.

Derepression of an IAP-inhibited caspase can also be detected as disassociation of an IAP-caspase species. An IAP-inhibited caspase can be identified as a caspase having an associated IAP using binding assays  
5 known in the art. Such a complex can be identified according to molecular weight or size using, for example, non-denaturing polyacrylamide gel electrophoresis, size exclusion chromatography, or analytical centrifugation. An IAP-caspase complex can also be identified using a co-  
10 precipitation technique. For example, an IAP-caspase complex can be identified due to the ability of an antibody to co-precipitate with both partners but not with one or the other partner alone. Similar, techniques can be used when either the IAP or caspase has been modified  
15 by a recombinant DNA method to incorporate an affinity tag such as glutathione-S-transferase (Amersham Pharmacia; Piscataway, NJ), which can be precipitated with glutathione beads; polyhistidine tag (Qiagen; Chatsworth, CA), which can be precipitated with Nickel NTA sepharose;  
20 antibody epitopes such as the flag peptide (Sigma; St Louis, MO), which can be immunoprecipitated; or other known affinity tag. An agent that prevents IAP-caspase complex formation or otherwise causes dissociation of the complex can be identified in such an assay as a  
25 derepressor of an IAP-inhibited caspase.

The caspases are present in cells as precursor polypeptides referred to as procaspases. Caspase activation occurs due to proteolytic processing of the procaspase. For example, caspase-3 is a heterotetramer  
30 composed of approximately 17-20 kDa and 11 kDa polypeptides that are formed by proteolysis of a 32 kDa polypeptide precursor, pro-caspase-3. Cleavage of the

pro-caspase-3 proceeds in two steps. The first cleavage results in production of a partially processed large subunit (22-24 kDa) that still contains the pro-domain, and a smaller, fully processed, subunit of about 11 kDa.

5 In the second step, the pro-domain is cleaved from the partially processed large subunit, probably by an autocatalytic process, to produce the 17-20 kDa mature, fully processed large subunit of the caspase-3 enzyme. Removal of the pro-domain, however, is not necessary for  
10 protease activation, as the partially processed caspase also has caspase activity.

The methods of the invention for identifying an agent that derepresses an IAP-inhibited caspase can be used to identify a caspase that is prevented from being  
15 processed to a mature, fully proteolytically active form due to the presence of an IAP. For example, the methods can be used to identify an agent that prevents or suppresses an IAP from inhibiting processing of a procaspase to a caspase. Because processing of a  
20 procaspase to a caspase will coincide with an increase in caspase proteolytic activity, the methods described above for determining proteolytic activity can be used in a method for identifying an agent that prevents or suppresses an IAP from inhibiting processing of a  
25 procaspase to a caspase. Similarly, a binding assay, such as those described above, can be used to identify a procaspase-IAP complex according to the combined molecular weight of the partners. An agent that prevents complex formation or causes the complex to dissociate can be  
30 identified in such an assay as a derepressor of an IAP-inhibited caspase. A caspase that is prevented from being processed to a mature, fully proteolytically active form



due to the presence of an IAP can also be identified according to differences in molecular weight or size of the mature and procaspase forms. Thus, an agent that, when contacted with a procaspase in the presence of an inhibitory IAP, causes a change in molecular weight or size indicative of the mature form can be identified as a derepressor of an IAP-inhibited caspase.

The methods of the invention can be used to identify a derepressor of an IAP-inhibited caspase that has specificity for a particular IAP or caspase or combination of a particular IAP and caspase. For example, the invention provides screening assays for identifying agents that alter the specific binding of a eukaryotic IAP such as XIAP, c-IAP-1 or c-IAP-2 and a caspase such as caspase-3, caspase-7 or caspase-9. Any IAP, including any eukaryotic IAP, can be used in a method of the invention in combination with the appropriate caspase. Other IAP proteins that are involved in regulating particular caspases can be identified using the methods disclosed herein, then the particular combination of caspase and IAP can be used in a screening assay to identify an agent that modulates the regulation of caspase activation by the IAP or that alters the specific association of the IAP and caspase.

As disclosed herein, invention core peptides were identified by screening combinatorial libraries having core tetrapeptide and hexapeptide structures. In view of the disclosed methods, the skilled artisan would recognize that combinatorial libraries of peptides having more than six amino acids or less than four amino acids also can be screened to identify other core peptides that

derepress an IAP-inhibited caspase. Furthermore, while the disclosed methods can be used to initially identify core peptides that derepress an IAP-inhibited caspase, those skilled in the art would know that the methods can  
5 be used in an iterative fashion to optimize or to identify additional core peptides that derepress an IAP-inhibited caspase, as described below.

It is expected that those skilled in the art can use combinatorial synthetic methods coupled to rapid  
10 screening methods to optimize and identify additional derepressors with increased binding affinity for an IAP or increased activity in derepressing an IAP-inhibited caspase, thereby possessing enhanced therapeutic potential.

15 The iterative approach is well-known in the art and is set forth, in general, in Houghten *et al.*, Nature, 354, 84-86 (1991); and Dooley *et al.*, Science, 266, 2019-2022 (1994); both of which are incorporated herein by reference. In the iterative approach, for example,  
20 sublibraries of a molecule having three variable groups are made wherein the first variable is defined. Each of the compounds with the defined variable group is reacted with all of the other possibilities at the other two variable groups. These sub-libraries are each tested to  
25 define the identity of the second variable in the sub-library having the highest activity in the screen of choice. A new sub-library with the first two variable positions defined is reacted again with all the other possibilities at the remaining undefined variable  
30 position. As before, the identity of the third variable position in the sub-library having the highest activity is

determined. If more variables exist, this process is repeated for all variables, yielding the compound with each variable contributing to the highest desired activity in the screening process. Promising compounds from this  
5 process can then be synthesized on larger scale in traditional single-compound synthetic methods for further biological investigation.

The positional-scanning approach has been described for various organic libraries and for various  
10 peptide libraries (see, for example, R. Houghten *et al.* PCT/US91/08694 and U.S. Patent 5,556,762, both of which are incorporated herein by reference). In the positional scanning approach sublibraries are made defining only one variable with each set of sublibraries and all possible  
15 sublibraries with each single variable defined (and all other possibilities at all of the other variable positions) is made and tested. From the instant description one skilled in the art could synthesize libraries wherein 2 fixed positions are defined at a time.  
20 From the testing of each single-variable defined library, the optimum substituent at that position is determined, pointing to the optimum or at least a series of compounds having a maximum of the desired biological activity. Thus, the number of sublibraries for compounds with a  
25 single position defined will be the number of different substituents desired at that position, and the number of all the compounds in each sublibrary will be the product of the number of substituents at each of the other variables.

30 Phage display methods provide a means for expressing a diverse population of random or selectively

randomized peptides. Various methods of phage display and methods for producing diverse populations of peptides are well known in the art. For example, Ladner et al. (U.S. Patent No. 5,223,409, issued June 29, 1993) describe  
5 methods for preparing diverse populations of binding domains on the surface of a phage. In particular, Ladner et al. describe phage vectors useful for producing a phage display library, as well as methods for selecting potential binding domains and producing randomly or  
10 selectively mutated binding domains.

An invention derepressor of an IAP-inhibited caspase that contains peptide moieties can be synthesized using amino acids, the active groups of which are protected as required using, for example, a  
15 t-butyldicarbonate (t-BOC) group or a fluorenylmethoxy carbonyl (Fmoc) group. Amino acids and amino acid analogs can be purchased commercially (Sigma Chemical Co., St. Louis MO; Advanced Chemtec, Louisville KY) or synthesized using methods known in the art. Peptides synthesized  
20 using the solid phase method can be attached to a variety of resins, including, for example, 4-methylbenzhydrylamine (MBHA), 4-(oxymethyl)-phenylacetamido methyl and 4-(hydroxymethyl) phenoxymethyl-copoly(styrene-1% divinylbenzene (Wang resin), all of which are commercially  
25 available, or to p-nitrobenzophenone oxime polymer (oxime resin), which can be synthesized as described by De Grado and Kaiser, J. Org. Chem. 47:3258 (1982).

The choice of amino acids or amino acid analogs incorporated into an invention peptide will depend, in  
30 part, on the specific physical, chemical or biological characteristics required of the derepressor of an IAP-

inhibited caspase. Such characteristics are determined by whether, for example, the peptide is to be used *in vivo* or *in vitro*, and, when used *in vivo*, by the route by which the invention peptide will be administered or the location  
5 in a subject to which it will be directed. For example, the derepressor of IAP-inhibited caspase core peptides exemplified herein can be synthesized using only L-amino acids. However, the skilled artisan would know that any or all of the amino acids in a peptide of the invention  
10 can be a naturally occurring L-amino acid, a non-naturally occurring D-amino acid or an amino acid analog, provided the peptide can derepress an IAP-inhibited caspase.

The choice of including an L-amino acid or a D-amino acid in the invention peptides depends, in part, on  
15 the desired characteristics of the peptide. For example, the incorporation of one or more D-amino acids can confer increased stability on the peptide *in vitro* or *in vivo*. The incorporation of one or more D-amino acids also can increase or decrease the activity, such as IAP binding  
20 affinity, of the peptide as determined, for example, using the assay described herein in Example VII or other well known methods for determining the binding affinity of a particular peptide to a particular protein.

As set forth above, invention peptides can be  
25 either linear, cyclic or multivalent, and the like, which conformations can be achieved using methods well-known in the art. As used herein a "cyclic" peptide refers to analogs of synthetic linear peptides that can be made by chemically converting the structures to cyclic forms.  
30 Cyclization of linear peptides can modulate bioactivity by increasing or decreasing the potency of binding to the

target protein (Pelton, J.T., et al., Proc. Natl. Acad. Sci., U.S.A., 82:236-239). Linear peptides are very flexible and tend to adopt many different conformations in solution. Cyclization acts to constrain the number of conformations available in solution, and thus, can favor a conformation having a higher affinity for IAP or more potent activity as a derepressor of an IAP-inhibited caspase.

Cyclization of linear peptides is accomplished either by forming a peptide bond between the free N-terminal and C-terminal ends (homodetic cyclopeptides) or by forming a new covalent bond between amino acid backbone and/or side chain groups located near the N- or C-terminal ends (heterodetic cyclopeptides) (Bodanszky, N., 1984, *supra*). The latter cyclizations use alternate chemical strategies to form covalent bonds, e.g. disulfides, lactones, ethers, or thioethers. Linear peptides of five or more amino acid residues, as described herein, can be cyclized relatively easily. The propensity of the peptide to form a beta-turn conformation in the central four residues facilitates the formation of both homo- and heterodetic cyclopeptides. The presence of proline or glycine residues at the N- or C-terminal ends also facilitates the formation of cyclopeptides, especially from linear peptides shorter than six residues in length.

An agent of the invention can be multivalent with respect to the number of derepressor IAP-inhibited caspase sequences or moieties are present per molecule. The sequences or moieties present in a multivalent agent can be either the same or different. Exemplary multivalent peptides can be produced using the well-known

multiple antigen peptide system (MAPS; see, e.g., Briand et al., 1992, J. Immunol Meth., 156(2):255-265; Schott et al., 1996, Cell Immun., 174(2):199-209, and the like). An agent that is multivalent with respect to the number of  
5 derepressor IAP-inhibited caspase sequences or moieties present can be useful for interacting with an IAP having more than one BIR domain. For example, a single agent can be made to contain two or more sequences or moieties that interact with separate BIR domains on the same IAP. The  
10 presence of multiple interacting partners in the multivalent agent and IAP can increase affinity or specificity of the interaction.

In some cases, it can be desirable to allow a derepressor of an IAP-inhibited caspase to remain active  
15 for only a short period of time. In those cases, the incorporation of one or more L-amino acids in the agent can allow, for example, endogenous peptidases in a subject to digest the agent *in vivo*, thereby limiting the subject's exposure to the derepressor. In one embodiment,  
20 the agent, whether based on a peptide backbone or other structure, can include a peptide linkage through an L-aspartate moiety or residue. Degradation of the L-aspartate containing agent by the caspases that it derepresses can provide a feedback control mechanism  
25 minimizing the extent of apoptosis allowed by the agent. The skilled artisan can determine the desirable characteristics required of an invention agent by taking into consideration, for example, the age and general health of a subject, and the like. The half life in a  
30 subject of a peptide having, for example, one or more D-amino acids substituted for a corresponding L-amino acid

can be determined using methods well known to those in the field of pharmacology.

Selective modification of the reactive groups in a peptide also can impart desirable characteristics to a derepressor of an IAP-inhibited caspase. An invention peptide can be manipulated while still attached to the resin to obtain, for example, an N-terminal modified peptide such as an N-acetylated peptide. Alternatively, the peptide can be removed from the resin using hydrogen fluoride or an equivalent cleaving reagent and then modified. Agents synthesized containing the C-terminal carboxy group (Wang resin) can be modified after cleavage from the resin or, in some cases, prior to solution phase synthesis. Methods for modifying the N-terminus or C-terminus of a peptide are well known in the art and include, for example, methods for acetylation of the N-terminus and methods for amidation of the C-terminus.

Also encompassed within the scope of invention peptides are peptide analogs. As used herein, the term "peptide analog" includes any peptide having an amino acid sequence substantially the same as a sequence specifically shown herein, such as Core peptides 1 through 55, in which one or more residues have been conservatively substituted with a functionally similar residue and which displays the ability to functionally mimic an invention lectin-binding peptide as described herein. Examples of conservative substitutions include the substitution of one non-polar (hydrophobic) residue such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between



glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another.

5           As used herein the phrase "conservative substitution" also includes the use of a chemically derivatized residue in place of a non-derivatized residue, provided that such peptide displays the required IAP binding or inhibiting activity. A chemical derivative can  
10 include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form  
15 salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical  
20 derivatives are those peptides which contain one or more naturally occurring amino acid derivatives of the twenty standard amino acids. For examples: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substituted for lysine; 3-methylhistidine may be  
25 substituted for histidine; homoserine may be substituted for serine; and ornithine may be substituted for lysine. Peptides of the present invention also include any peptide having one or more additions, deletions or combination of additions and deletions of residues, relative to the  
30 sequence of a peptide whose sequence is shown herein, so long as the required IAP binding or inhibiting activity is maintained.

Those skilled in the art will recognize from the guidance provided herein that an agent of the invention can include a core peptide or core structure that is modified, derivatized, or substituted with an analogs or derivative so long as the agent is capable of derepressing an IAP-inhibited caspase. Such alterations in a core peptide or core structure can be made by well known synthetic methods such as those described herein. An agent so altered can be tested for activity using the methods described herein such as the derepression assay described in Example I or the polarization binding assay described in Example VII.

An agent identified as a derepressor of an IAP-inhibited caspase can be tested using an assay for determining caspase proteolytic activity or binding of IAP and caspase in the presence or absence of the agent including, for example, the assays described above. An agent that is identified as capable of derepressing an IAP-inhibited caspase using such assays can be further combinatorialized at one or more positions using the iteration approach described above. Alternatively, an identified derepressor or plurality of derepressors can be used as a basis for the rational design of second generation agents. For example, common structural features between a plurality of validated agents can be used to guide the synthesis of a generalized structure incorporating those shared features. Structural information regarding an agent when bound to an IAP or caspase can also be used to design a second generation agent that retains or improves upon moieties identified as providing favorable interactions while removing moieties

that lead to unfavorable interactions with the caspase or IAP.

The invention provides structure activity relationship (SAR) information of polyphenylurea compounds which is used in designing optimized second generation agents (see Example XVI and Table X and XI). A series of compounds, shown in Figure 34 (TPI 1509), were synthesized based on the TPI 1396 polyphenylurea compounds. All of these compounds were active in the XIAP derepression assay. Therefore, the invention provides an isolated agent having a core structure selected from any of the structures shown in Figure 34 where the agent derepresses an IAP-inhibited caspase. In addition, modifications of R groups of a compound from the TPI 1509 series are provided herein in Example XVII (see also Figure 35). As understood by one skilled in the art, these types of R group modifications can be used for other polyphenylurea compounds such as those in the TPI 1396 library.

The invention provides a method of identifying an agent that derepresses an IAP-inhibited caspase. The method includes the steps of (a) detecting a labeled derepressor of an IAP-inhibited caspase bound to an IAP or caspase; (b) contacting the bound IAP or caspase with a candidate agent, the candidate agent suspected of being able to derepress an IAP-inhibited caspase; and (c) detecting dissociation of the labeled derepressor of an IAP-inhibited caspase from the IAP or caspase, whereby the candidate agent is identified as an agent that derepresses an IAP-inhibited caspase. A labeled derepressor of an IAP-inhibited caspase used in the method can have a core motif selected from a core peptide of the invention such

as Core peptides 4 through 39 and 42 through 55 or a core structure selected from TPI 759, TPI 882, TPI 914, TPI 927, or a compound having a structure selected from TPI 1391, TPI 1349, TPI 1400, TPI 1396, TPI 1509, TPI 1540, 5 TPI 792 and TPI 1332. The methods can be used to identify a better derepressor of an IAP-inhibited caspase in a screening format as described above and in the Examples.

The invention also provides a novel negative regulatory binding site on an IAP. The novel negative 10 regulatory binding site identified herein does not bind to the SMAC peptide (also known as DIABLO). The SMAC peptide is known to bind to IAPs such as XIAP through the BIR3 domain of the IAP (Liu et al, Nature 408:1004-1008 (2000)). As disclosed herein in Figure 26, an active 15 polyphenylurea compound such as TPI 1396-34 does not compete with biotin-SMAC 7-mer peptide AVPIAQK (SEQ ID NO: 5) for binding to XIAP. As further disclosed herein in Example XVII and Figure 36, an active tetrapeptide compound such as TPI 1332-69 or TPI 1332-4 does not 20 compete with biotin-SMAC 7-mer peptide AVPIAQK (SEQ ID NO: 5) for binding to XIAP. Therefore, disclosed herein are compounds that act through a non-SMAC binding site on an IAP such as XIAP. As is further disclosed herein, the non-SMAC binding site of XIAP has been identified to be 25 the BIR2 domain. The ability of polyphenylurea compounds of the invention to derepress XIAP BIR2 domain-inhibited caspase is disclosed herein, for example, in Figures 21C, 22E, 23E and 24G. In addition, the ability of polyphenylurea compounds of the invention to bind directly 30 to BIR2 is shown in Figure 42 and described in Example XXIV.

These results indicate that agent that derepresses an IAP-inhibited caspase can function by binding to a BIR domain of the IAP and thereby reducing the ability of the BIR domain to block caspase-IAP function. Therefore, the invention provides a method of identifying an agent that derepresses an XIAP-inhibited caspase that involves (a) contacting a caspase and an BIR domain, wherein the BIR domain is capable of inhibiting the caspase, under conditions that allow caspase activity in the absence of the BIR domain, with a candidate agent, and (b) detecting caspase activity, wherein an increase in the activity of the inhibited caspase identifies an agent that derepresses an IAP-inhibited caspase.

The method of the invention for identifying an agent that derepresses an IAP-inhibited caspase involves contacting a caspase with a BIR domain that is capable of inhibiting the caspase. Any BIR domain that is capable of inhibiting a caspase can be used in the methods of the invention. The ability of a BIR domain to inhibit caspase activity can be determined using a variety of well known methods, for example, by determining a lower level of hydrolysis of a specific substrate by the caspase in the presence of the BIR domain as compared to the activity in the absence of the BIR domain. Given the role of caspases in apoptosis, it will be recognized by those skilled in the art that caspase activity can be identified directly, for example, by examining proteolysis (hydrolysis) of a specific substrate or indirectly, for example, by identifying morphological changes in a cell or cell nucleus characteristic of apoptosis. Exemplary assays for detecting caspase activity are described herein above and in Example I. An example of a BIR domain capable of

inhibiting caspase-3 is the BIR2 domain of XIAP (see Example VIII).

The invention further provides a method of identifying an agent that derepresses an IAP-inhibited caspase by (a) detecting a labeled derepressor of an IAP-inhibited caspase bound to a non-SMAC binding site on the IAP; (b) contacting the bound IAP or caspase with a candidate agent, the candidate agent suspected of being able to derepress an IAP-inhibited caspase; and (c) detecting dissociation of the labeled derepressor of an IAP-inhibited caspase from the IAP or caspase, whereby the candidate agent is identified as an agent that derepresses an IAP-inhibited caspase. In one embodiment, the labeled derepressor is based on a core structure from the TPI 1332 or TPI 1396 library. In another embodiment, the non-SMAC binding site on the IAP is a site bound by TPI 1332-69 or TPI 1332-4.

In a further embodiment, the non-SMAC binding site on the IAP is a BIR domain. Therefore, the method can be practiced by (a) detecting a labeled derepressor of a BIR domain-inhibited caspase, the derepressor bound to the BIR domain of a BIR domain-caspase complex; (b) contacting the BIR domain-caspase complex with a candidate agent, the candidate agent suspected of being able to derepress a BIR domain-inhibited caspase, and (c) detecting dissociation of the labeled derepressor of the BIR domain-inhibited caspase from the complex, wherein the derepressor is selected from an isolated agent comprising a core structure selected from TPI 1391, TPI 1349, TPI 1396, TPI 1509, TPI 1540, TPI 1400, TPI 792 and

TPI 1332, whereby the candidate agent is identified as an agent that derepresses an IAP-inhibited caspase.

As is described in Example XXIV, a compound of the invention that derepresses an XIAP-inhibited caspases  
5 can bind directly to the BIR2 domain of XIAP. It is recognized that an agent capable of competing with a compound of the invention that binds to a BIR2 domain will also bind to the BIR2 domain at a site important for derepression activity. Therefore, the invention provides  
10 a method for identifying an agent that derepresses an IAP-inhibited caspase based on the ability of the agent to compete with a compound of the invention for binding to a BIR2 domain. The method involves (a) contacting a BIR2 domain with a candidate agent in the presence of a  
15 derepressor of an IAP-inhibited caspase, under conditions wherein the BIR2 domain binds to the derepressor, and (b) detecting dissociation of the derepressor from the BIR2 domain, whereby the candidate agent is identified as an agent that derepresses an IAP-inhibited caspase, wherein  
20 the derepressor is selected from an isolated agent comprising a core structure selected from TPI 1391, TPI 1349, TPI 1396, TPI 1509, TPI 1540, TPI 1400, TPI 792 and TPI 1332.

A variety of assays are well known in the art  
25 that can be used to identify an agent that derepresses an IAP-inhibited caspase. Such methods include binding assays where candidate agents are added to a complex that contains a derepressor and an IAP such as XIAP. The derepressor or IAP can be immobilized, for example to a  
30 latex bead or plate or can be free in solution. The derepressor, IAP or candidate agent can be conjugated to a

radiolabel, fluorescent label or enzyme label such as alkaline phosphatase, horse radish peroxidase or luciferase. For example, a candidate agent can be added to a complex which contains an IAP and a labeled  
5 derepressor, for example, where the IAP is immobilized on a solid support such as a latex bead. The amount of labeled derepressor which is displaced by the candidate agent can then be determined. Alternatively, this assay can be performed where the IAP is not bound to a solid  
10 support but is free in solution. In addition, fluorescently labeled candidate compounds can also be added to a complex that contains a derepressor and IAP and bound complexes that contain the labeled candidate agent can be detected, for example, using a fluorescence  
15 polarization assay (Degterev et al., Nature Cell Biology 3:173-182 (2001)).

One skilled in the art understands that a variety of additional means can be used to determine whether a candidate agent is an agent that derepresses an  
20 IAP-inhibited caspase or whether the candidate agent can displace a derepressor bound to an IAP. For example, a scintillation proximity assay (Alouani, Methods Mol. Biol. 138:135-41 (2000)) can be used. Scintillation proximity assays involve the use of a fluomicrosphere coated with an  
25 acceptor molecule, such as an antibody, to which an antigen will bind selectively in a reversible manner. For example, an IAP-derepressor complex can be bound to a fluomicrosphere using an antibody that specifically binds to the IAP, and contacted with a  $^3\text{H}$  or  $^{125}\text{I}$  labeled FP  
30 candidate agent. If the labeled candidate agent specifically binds to the IAP, the radiation energy from the labeled candidate agent is absorbed by the



fluomicrosphere, thereby producing light which is easily measured.

Additional assays suitable for identifying an agent that derepresses an IAP-inhibited caspase and for  
5 determining specific binding of a candidate agent to an XIAP after displacing a derepressor can include, without limitation, UV or chemical cross-linking assays (Fancy, Curr. Opin. Chem. Biol. 4:28-33 (2000)) and biomolecular interaction analyses (Weinberger et al., Pharmacogenomics  
10 1:395-416 (2000)). Specific binding of a candidate agent to an IAP can be determined by cross-linking these two components, if they are in contact with each other, using UV or a chemical cross-linking agent. In addition, a biomolecular interaction analysis (BIA) can detect whether  
15 two components are in contact with each other. In such an assay, one component, such as an IAP-derepressor complex is bound to a BIA chip, and a second component such as a candidate agent is passed over the chip. If the candidate agent displaces the derepressor and binds to the IAP, the  
20 contact results in an electrical signal, which is readily detected.

Further assays suitable for identifying an agent that derepresses an IAP-inhibited caspase include those based on NMR methods. Such methods take advantage of the  
25 significant perturbations that can be observed in NMR-sensitive parameters of a candidate agent or its target, such as an IAP or domain thereof, that occur upon complex formation between the agent and target. These perturbations can be used to detect binding between a  
30 candidate agent and IAP, as well as to assess the strength of the binding interaction. In addition, some NMR

techniques allow the identification of the agent binding site or which part of the agent is responsible for interacting with the target. Exemplary NMR methods useful for identifying an agent that derepresses an IAP-inhibited caspase include "SAR by NMR," which is described, for example, in Shuker et al. *Science*, 274, 1531-1534 (1996), and a variety of NMR-based screening assays, including SHAPES screening, fragment-based approaches for lead optimization using NMR, and fluorine-NMR competition binding experiments, all of which are described, for example, in Combinatorial Chemistry & High Throughput Screening, Vol. 5, No. 8 (2002) and in Hajduk et al., Quarterly Reviews of Biophysics 32(3):211-240 (1999).

Fluorescence-based assays are also suitable for identifying an agent that derepresses an IAP-inhibited caspase. Examples of fluorescence methods applicable to determining an interaction between an agent that derepresses an IAP-inhibited caspase and its corresponding target, such as an IAP or caspase, include observations of fluorescence intensity changes resulting from an alteration in interaction between agent and target; fluorescence resonance energy transfer (FRET), which is useful for determining change in fluorescence intensity based on distance between agent and target; fluorescence polarization changes resulting a change in size of an observed binding partner when associated or dissociated from the another binding partner; fluorescence lifetime changes, and fluorescence correlation spectroscopy, which is based on translation diffusion, a parameter related to the size of an observed binding partner. Such methods can involve employing a fluorescently labeled agent or binding partner. For example, a fluorophore can be detected based

on the excitation or emission wavelengths of the fluorophore, fluorescence polarization of the fluorophore, or intensity of fluorescence emitted from the fluorophore. Alternatively, detection can be based on a difference in a measurable property of the label for the bound and unbound state. For example, as demonstrated in Example VII, difference in fluorescence polarization due to the slower rotation of a substrate bound to an IAP compared to the unbound substrate can be used to detect association.

Other measurable differences that can be used to determine association of a fluorophore-labeled agent with an IAP or caspase include, for example, different emission intensity due to the presence or absence of a quenching agent, difference in emission wavelength due to the presence or absence of a fluorescence resonance energy transfer (FRET) donor or acceptor, or difference in emission wavelength due to differences in fluorophore conformation or environment. A derepressor of an IAP-inhibited caspase used in a method of the invention can be labeled with any of a variety of labels including, for example, those described above. A labeled derepressor that is bound to an IAP or caspase can be detected according to a known measurable property of the label.

Dissociation of the labeled derepressor of an IAP-inhibited caspase from the IAP or caspase can be detected as absence or reduction in the amount of label from the IAP or caspase in the presence of a competitive binding candidate agent or as a reversal of a change that occurs upon association of the labeled agent with a caspase or IAP in the presence of a competitive binding candidate agent. Thus, dissociation can be detected in the presence of a non-labeled candidate agent as a

reduction or loss of radioactivity of the IAP or caspase in the presence of a radionucleotide labeled derepressor, reduction or loss of electromagnetic absorbance at a specified wavelength for the IAP or caspase in the presence of a chromophore labeled derepressor, reduction or loss of magnetic signal at a specified field strength or radio frequency for the IAP or caspase in the presence of a paramagnetic spin labeled derepressor or reduction or loss of a secondary label associated with the IAP or caspase in the presence of a derepressor that is labeled with a binding group for the secondary label. An example of dissociation measured by the reversal of a change occurring upon association is provided in Example VII, where a difference in polarization due to the faster rotation of a dissociated substrate compared to the IAP-bound substrate is used to detect dissociation.

Other changes in a property of a label that can be detected to determine association or dissociation of an appropriately labeled derepressor and IAP or caspase include, for example, absorption and emission of heat, absorption and emission of electromagnetic radiation, affinity for a receptor, molecular weight, density, mass, electric charge, conductivity, magnetic moment of nuclei, spin state of electrons, polarity, molecular shape, or molecular size. Properties of the surrounding environment that can change upon association or dissociation of an appropriately labeled derepressor and IAP or caspase include, for example, temperature and refractive index of surrounding solvent. Association and dissociation of a derepressor from an IAP or caspase can be measured based on any of a variety of properties of a labeled derepressor or of the complex between a derepressor and IAP or caspase

using well known methods including, for example, equilibrium binding analysis, competition assays, and kinetic assays as described in Segel, Enzyme Kinetics John Wiley and Sons, New York (1975), and Kyte, Mechanism in  
5 Protein Chemistry Garland Pub. (1995).

In addition, virtual computational methods and the like can be used to identify compounds that can displace a derepressor in a screening method of the invention. Exemplary virtual computational methodology  
10 involves virtual docking of small-molecule agents on a virtual representation of an IAP or IAP-derepressor complex structure in order to determine or predict specific binding. See, for example, Shukur et al., *supra*, 1996; Lengauer et al., Current Opinions in Structural  
15 Biology 6:402-406 (1996); Choichet et al., Journal of Molecular Biology 221:327-346 (1991); Cherfils et al., Proteins 11:271-280 (1991); Palma et al., Proteins 39:372-384 (2000); Eckert et al., Cell 99:103-115 (1999); Loo et al., Med. Res. Rev. 19:307-319 (1999); Kramer et  
20 al., J. Biol. Chem. (2000).

The methods of the invention for identifying an agent that derepresses an IAP-inhibited caspase can be performed using low throughput or high throughput assay formats. Screening can be carried out in all plate  
25 formats, including for example, 96, 384 and 1536 well formats. In addition, assays such as those described above can be performed in kinetic-based or end point-based formats. To increase screening throughout, more than one candidate agent or caspase can be present in an assay  
30 sample. The number of different candidate agents to test in the methods of the invention will depend on the

application of the method. For example, one or a small number of candidate agents can be screened using manual screening procedures, or when it is desired to compare efficacy among several candidate agents. However, it will be appreciate that the larger the number of candidate agents, the greater the likelihood of identifying an agent having the desired activity in a screening assay. Additional, large numbers of candidate agents can be processed in high-throughput automated screening methods.

10

The invention further provides a method for identifying a derepressor of an IAP-inhibited caspase in a database. A database of molecules such as peptides or small molecules can be queried with the structure of a derepressor of an IAP-inhibited caspase to identify candidate agents having a moiety identical or similar to the query structure. A candidate agent identified in a database search can be synthesized, isolated or otherwise obtained using known methods and then tested for its level of activity as a derepressor of an IAP-inhibited caspase using the assays described above and in the Examples.

For peptide based derepressors, a query can be made to a database based on amino acid sequence (primary structure) or three dimensional structure (tertiary structure) or a combination of both to identify peptides or proteins having identical or substantially similar structures. Methods for comparing primary sequence structure which can be used to determine that two sequences are substantially the same are well known in the art as are databases including, for example, SwissProt and GenPept. For example, one method for determining if two

30

sequences are substantially the same is BLAST, Basic Local Alignment Search Tool, which can be used according to default parameters as described by Tatiana et al., FEMS Microbial Lett. 174:247-250 (1999) or on the National Center for Biotechnology Information web page. BLAST is a set of similarity search programs designed to examine all available sequence databases and can function to search for similarities in amino acid or nucleic acid sequences. A BLAST search provides search scores that have a well-defined statistical interpretation. Furthermore, BLAST uses a heuristic algorithm that seeks local alignments and is therefore able to detect relationships among sequences which share only isolated regions of similarity including, for example, protein domains (Altschul et al., J. Mol. Biol. 215:403-410 (1990)).

In addition to the originally described BLAST (Altschul et al., *supra*, 1990), modifications to the algorithm have been made (Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997)). One modification is Gapped BLAST, which allows gaps, either insertions or deletions, to be introduced into alignments. Allowing gaps in alignments tends to reflect biologic relationships more closely. For example, gapped BLAST can be used to identify sequence identity within similar domains of two or more polypeptides. A second modification is PSI-BLAST, which is a sensitive way to search for sequence homologs. PSI-BLAST performs an initial Gapped BLAST search and uses information from any significant alignments to construct a position-specific score matrix, which replaces the query sequence for the next round of database searching. A PSI-BLAST search is often more sensitive to weak but biologically relevant sequence similarities.

A second resource that can be used to determine if two sequences are substantially the same is PROSITE, available on the world wide web at ExpASy. PROSITE is a method of determining the function of uncharacterized polypeptides translated from genomic or cDNA sequences (Bairoch et al., Nucleic Acids Res. 25:217-221 (1997)). PROSITE consists of a database of biologically significant sites and patterns that can be used to identify which known family of polypeptides, if any, the new sequence belongs. Using this or a similar algorithm, a polypeptide that is substantially the same as another polypeptide can be identified by the occurrence in its sequence of a particular cluster of amino acid residues, which can be called a pattern, motif, signature or fingerprint, that is substantially the same as a particular cluster of amino acid residues in a reference polypeptide including, for example, those found in similar domains. PROSITE uses a computer algorithm to search for motifs that identify polypeptides as family members. PROSITE also maintains a compilation of previously identified motifs, which can be used to determine if a newly identified polypeptide is a member of a known family.

Tertiary structure of a derepressor of an IAP-inhibited caspase can be determined by a theoretical method such as *ab initio* protein folding using algorithms known in the art or by an empirical method such as X-ray crystallographic or nuclear magnetic resonance based structure determination. A structural model of a derepressor can be used in an algorithm that compares polypeptide structure including, for example, SCOP, CATH, or FSSP which are reviewed in Hadley and Jones, Structure 7:1099-1112 (1999) and regions having a particular fold or



conformation used as a region for sequence comparison to a second polypeptide to identify substantially similar regions.

Similar database searching methods can be used  
5 for non-peptide based derepressors or to query a database  
of non-peptide based candidate agents based on structure.  
A database can be searched, for example, by querying based  
on chemical property information or on structural  
information. In the latter approach, an algorithm based  
10 on finding a match to a template can be used as described,  
for example, in Martin, "Database Searching in Drug  
Design," J. Med. Chem. 35:2145-2154 (1992).

A derepressor of an IAP-inhibited caspase can  
also be identified in a database using the results of a  
15 positional scanning synthetic combinatorial library as a  
query. Such results can be represented as a motif and the  
motif used to search a database for a derepressor of an  
IAP-inhibited caspase. Motif searches are generated from  
screening results of positional scanning synthetic  
20 combinatorial libraries, and contained in each position  
are amino acids corresponding to mixtures having an  
activity threshold greater than a specified value. An  
example of an activity threshold is the ratio of  $V_{\max}$  for  
caspase activity in the presence and absence of a  
25 candidate agent as described in Example I. Motif based  
database searching is known in the art as described, for  
example, in Hemmer et al., Nat. Med. 5:1375-1382 (1999),  
Hemmer et al., J. Exp. Med. 185:1651-1659 (1997) and  
Hemmer et al., Immunol Today 19:163-168 (1998).

Alternatively, results from a positional scanning synthetic combinatorial library can be represented as a score matrix and the score matrix used to query for other derepressors of an IAP-inhibited caspase in a sequence database. Methods for identifying candidate peptides or proteins based on score-matrix based searches of a databases are described in Zhao et al., J. Immunol. 167:2130-2141 (2001). Briefly, a matrix is constructed in which columns represent positions, rows represent the 20 amino acids and each is correlated with a score. The score for a particular position and amino acid is based on assay results for the mixture of a positional scanning synthetic combinatorial library corresponding to that amino acid defined at that position. For example, each score can correspond to the ratio of  $V_{\max}$  for caspase activity in the presence and absence of the mixture corresponding to the amino acid defined at the particular position. The scoring matrix is then used to search for candidate derepressors of an IAP-inhibited caspase by moving the scoring matrix across database entries in 1 amino acid increments. A score is calculated for the database entries searched and each is ranked. Those having a score above a predetermined cutoff are identified as candidate derepressors of an IAP-inhibited caspase.

The invention provides a method of derepressing an IAP-inhibited caspase, by contacting an IAP-inhibited caspase with an effective amount of an agent to derepress an IAP-inhibited caspase, the agent having a core motif selected from a core peptide of the invention, such as core peptides 4 through 39 and 42 through 55, or a core structure of the invention such as TPI 759, TPI 882, TPI 914 or TPI 927.

For inhibiting a caspase inhibitory activity of an inhibitor of apoptosis protein (IAP), the IAP-inhibited caspase is contacted with an amount of derepressor effective to derepress the IAP-inhibited caspase. Thus, 5 an effective amount of the agent is an amount that is sufficient to yield an increase in caspase proteolytic activity from the derepressed IAP-inhibited caspase compared to the caspase activity for an IAP-inhibited caspase. An increase in proteolytic activity from a 10 derepressed IAP-inhibited caspase can be determined using any of the methods described above in reference to a method for identifying a derepressor of an IAP-inhibited caspase.

An agent of the invention can be contacted with 15 an IAP-inhibited caspase under conditions suitable for caspase activity to occur once an IAP is inhibited from inhibiting the caspase. Such conditions include those described in Example I. The agent that is contacted with the IAP-inhibited caspase can be present in a mixture of 20 compounds, in an isolated form or in substantially pure form. As described above, a mixture of compounds can be contacted with an IAP-inhibited caspase in a screening method employing positional scanning or iteration. Such a mixture can be identified as having the ability to 25 derepress an IAP-inhibited caspase. The mixture can be used in the methods of the invention to derepress an IAP inhibited caspase. Alternatively, a particular species in the mixture having such activity can be further defined by isolating individual species in the mixture and repeating 30 the derepression assay or performing a second assay for derepression of an IAP-inhibited caspase. An agent that derepresses an IAP-inhibited caspase can be contacted with

the IAP-inhibited caspase in a substantially pure form, as a conjugate or in a formulation as described above.

In a further embodiment of the invention an IAP-inhibited caspase can be contacted with an agent of the invention in a cell. Accordingly, the invention provides a method of promoting apoptosis in a cell, by contacting the cell with an effective amount of an agent to derepress an IAP-inhibited caspase, the agent having a core motif selected from a core peptide of the invention, such as Core peptides 4 through 39 and 42 through 55, or a core structure of the invention such as TPI 759, TPI 882, TPI 914 or TPI 927.

Methods described herein for cytosolic delivery of an IAP-inhibited caspase, such as attachment of a moiety of conjugate, can be used in a method of promoting apoptosis in a cell. An effective amount of the agent can be identified as an amount sufficient to allow apoptosis to occur in the cell. Methods of determining morphological changes in a cell or nucleus that are characteristic of apoptosis, such as those described above in relation to identifying a derepressor of an IAP-inhibited caspase, can be used to monitor apoptosis while performing a method of promoting apoptosis in a cell.

The invention also provides a method for reducing the ability of a population of cells to survive *ex vivo*. The method can include the steps of contacting the cells with an agent of the invention, wherein the agent derepresses an IAP-inhibited caspase. The cells can be contacted with the agent using the methods described above for promoting apoptosis in a cell. The methods can

be used to remove a particular subpopulation of cells in a sample using the targeting methods described above, such as the attachment of a targeting moiety to the agent.

The methods of the invention can be carried out  
5 in a cell from any organism in which apoptosis can occur when an IAP-inhibited caspase is derepressed including, for example, a eukaryotic cell, such as a mammalian cell, human cell, non human-primate cell, mouse cell, hamster cell, or other animal cell; an invertebrate cell such as a  
10 fly or nematode cell or a yeast cell. Various cell types can be used in the methods of the invention including, for example, a tumor cell, stem cell, neural cell, fat cell, hematopoietic cell, liver cell or muscle cell. In particular the methods are useful for inducing apoptosis  
15 in aberrantly regulated cells including, for example, cells that exhibit uncontrolled cell proliferation as well as cells that exhibit dysfunction in specific phases of the cell cycle, leading to altered proliferative characteristics or morphological phenotypes. Specific  
20 examples of aberrantly regulated cell types include neoplastic cells such as cancer and hyperplastic cells characteristic of tissue hyperplasia. Another specific example includes immune cells that become aberrantly activated or fail to down regulate following stimulation.  
25 Autoimmune diseases are mediated by such aberrantly regulated immune cells. Aberrantly regulated cells also include cells that are biochemically or physiologically dysfunctional. Other types of aberrant regulation of cell function or proliferation are known to those skilled in  
30 the art and are similarly target cells of the invention applicable for apoptotic destruction using the methods of the invention.

Because a number of characteristic changes associated with apoptosis of a cell are due to the proteolytic activity of caspases, the methods can be used to induce characteristic changes of apoptosis. For  
5 example, caspase induced proteolysis of lamin B, which is involved in attachment of chromatin to the nuclear envelope, can be responsible for collapse of the chromatin associated with apoptosis (Martin and Green, *supra*, 1995). Caspase induced proteolysis of the 45 kDa subunit of DNA  
10 fragmentation factor (DFF-45) activates a pathway leading to fragmentation of genomic DNA into nucleosomal fragments (Liu et al., Cell 89:175-184 (1997)). In addition, caspase induced proteolysis of PARP can prevent the ability of PARP to repair DNA damage, further contributing  
15 to the morphologic changes associated with apoptosis. Thus, the methods of the invention can be used to induce collapse of the chromatin and fragmentation of genomic DNA associated with apoptosis. Other caspase target proteins include sterol regulatory element binding proteins;  
20 retinoblastoma (RB) protein; DNA-dependent kinase; U1 70-K kinase; and the large subunit of the DNA replication complex (Wang et al., EMBO J. 15:1012-1020 (1996); Takahashi et al., Proc. Natl. Acad. Sci., USA 93:8395-8400 (1996); Casciola-Rosen et al., J. Exp. Med. 183:1957-1964  
25 (1996); and Ubeda and Habener, J. Biol. Chem. 272:19562-19568 (1997)) each of which can be induced to be proteolyzed by the methods of the invention.

In mammalian cells, activation of caspases is achieved through at least two independent mechanisms,  
30 which are initiated by distinct caspases but result in activation of common "executioner" caspases. Apoptosis initiated by ligand binding to the Fas receptor is one

well described cell death pathway. In this pathway, binding of a ligand to Fas allows the intracellular domain of Fas to bind the intracellular MORT1 (FADD) protein, which, in turn, binds to caspase-8 (MACH; FLICE; Mch5; see  
 5 Boldin et al., Cell 85:803-815 (1996); Muzio et al., Cell 85:817-827 (1996)). These results define caspase-8 as an upstream caspase involved in the Fas cell death pathway. In addition, caspase-3 is activated in the Fas cell death pathway, suggesting that an upstream protease such as  
 10 caspase-8 or a protease activated by caspase-8 is involved in caspase-3 activation. Accordingly, the methods of the invention can be used to directly derepress IAP inhibited-caspase-8 thereby effectively derepressing the downstream caspase-3 protease.

15 Caspase activation also can involve cytochrome c, which in mammalian cells is often released from mitochondria into the cytosol during apoptosis (Liu et al., Cell 86:147-157 (1996); Kharbanda et al., Proc. Natl. Acad. Sci., USA 94:6939-6942 (1997); Kluck et al.,  
 20 Science 275:1132-1136 (1997); and Yang et al., Science 275:1129-1132 (1997)). Upon entering the cytosol, cytochrome c induces the ATP- or dATP-dependent formation of a complex of proteins that results in proteolytic activation of pro-caspase-3 and apoptotic destruction of  
 25 nuclei (Liu et al., *supra*, 1996). Among the members of this complex are the CED-4 homolog Apaf-1, and caspase-9 (Apaf-3; Liu et al., *supra*, 1996; Li et al., Cell 91:479-489 (1997); Zou et al., Cell 90:405-413 (1997)). XIAP, c-IAP-1 and c-IAP-2 suppress apoptosis induced by  
 30 stimuli known to cause release of cytochrome c from mitochondria and can inhibit caspase activation induced by cytochrome c *in vitro*. Thus, the agents and methods of

the invention can be used to allow apoptosis to occur in response to release of cytochrome c from mitochondria by suppressing inhibition of a caspase by XIAP, c-IAP-1 or c-IAP-2.

5           The invention further provides a method of reducing the severity of a pathologic condition in an individual, by administering to an individual having a pathologic condition characterized by a pathologically reduced level of apoptosis, an effective amount of an  
10 agent to derepress an IAP-inhibited caspase. Examples of conditions characterized by pathologically reduced levels of apoptosis that can be treated in a method of the invention include, but are not limited to, restenosis; autoimmune disease such as lupus or Rheumatoid Arthritis;  
15 allograft rejection, proliferative lesions of the skin such as Eczema; or benign prostate hypertrophy. The agent can have a core motif selected from a core peptide of the invention, such as Core peptides 4 through 39 and 42 through 55, or a core structure of the invention such as  
20 TPI 759, TPI 882, TPI 914, TPI 927 or a compound comprising a core structure selected from TPI 1391, TPI 1349, TPI 1396, TPI 1509, TPI 1540, TPI 1400, TPI 792 and TPI 1332.

          An effective amount of an agent that derepresses  
25 an IAP-inhibited caspase when used to treat a pathological condition is an amount required to allow an increase in apoptosis when administered to an individual. The dosage of an agent of the invention required to be therapeutically effective will depend, for example, on the  
30 pathological condition to be treated, the route and form of administration, the weight and condition of the



individual, and previous or concurrent therapies. The appropriate amount considered to be an effective dose for a particular application of the method can be determined by those skilled in the art, using the guidance provided  
5 herein. For example, the amount can be extrapolated from *in vitro* or *in vivo* assays as described previously. One skilled in the art will recognize that the condition of the patient can be monitored throughout the course of therapy and that the amount of the agent that is  
10 administered can be adjusted accordingly.

For treating or reducing the severity of a pathological condition, an effective amount is an efficacious amount of the agent capable of increasing apoptosis that is pathologically reduced. An effective  
15 amount can be, for example, between about 10  $\mu\text{g/kg}$  to 500  $\text{mg/kg}$  body weight, for example, between about 0.1  $\text{mg/kg}$  to 100  $\text{mg/kg}$ , or preferably between about 1  $\text{mg/kg}$  to 50  $\text{mg/kg}$ , depending on the treatment regimen. For example, if an agent or formulation containing the agent is  
20 administered from one to several times a day, then a lower dose would be needed than if a formulation were administered weekly, or monthly or less frequently. Similarly, formulations that allow for timed-release of the agent, such as those described above, would provide  
25 for the continuous release of a smaller amount of derepressor of apoptosis than would be administered as a single bolus dose. For example, an agent of the invention can be administered at between about 1-5  $\text{mg/kg/week}$ .

Formulations of a derepressor of an IAP-  
30 inhibited caspase, variants and combinations thereof can also be delivered in an alternating administrations so as

to combine their apoptosis increasing effects over time. For example, an agent having a core peptide or structure of the invention can be administered in a single bolus dose followed by multiple administrations of one or more  
5 such agents species or variant alone, or in combination with a different formulation of such an agent or formulation of a different agent. Whether simultaneous or alternating delivery of the agent formulation, variant or combination thereof, the mode of administration can be any  
10 of those types of administrations described previously and will depend on the particular therapeutic need and efficacy of the derepressor of an IAP-inhibited caspase selected for the purpose. Determining which agent, formulation, species and variants to combine in a  
15 temporally administered regime, will depend on the pathological condition to be treated and the specific physical characteristics of the individual affected with the disease. Those skilled in the art will know or can determine a specific regime of administration which is  
20 effective for a particular application using the teachings and guidance provided herein together with diagnostic and clinical criteria known within the field of art of the particular pathological condition.

The methods of treating a pathological condition  
25 characterized by pathologically reduced apoptosis additionally can be practiced in conjunction with other therapies. For example, for treating cancer, the methods of the invention can be practiced prior to, during, or subsequent to conventional cancer treatments such as  
30 surgery, chemotherapy, including administration of cytokines and growth factors, radiation or other methods known in the art.

Such treatments can act in a synergistic manner, with the reduction in tumor mass caused by the conventional therapy increasing the effectiveness of a compound of the invention, and vice versa. Non-limiting  
5 examples of anti-cancer drugs that are suitable for co-administration with a compound of the invention are well known to those skilled in the art of cancer therapy and include aminoglutethimide, amsacrine (m-AMSA), azacitidine, asparaginase, bleomycin, busulfan,  
10 carboplatin, carmustine (BCNU), chlorambucil, cisplatin (cis-DDP), cyclophosphamide, cytarabine HCl, dacarbazine, dactinomycin, daunorubicin HCl, doxorubicin HCl, erythropoietin, estramustine phosphate sodium, etoposide (V16-213), floxuridine, fluorouracil (5-FU), flutamide,  
15 hexamethylmelamine (HMM), hydroxyurea (hydroxycarbamide), ifosfamide, interferon alpha, interleukin 2, leuprolide acetate (LHRH-releasing factor analogue), lomustine (CCNU), mechlorethamine HCl (nitrogen mustard), melphalan, mercaptopurine, mesna, methotrexate (MTX), mitoguazone  
20 (methyl-GAG, methyl glyoxal bis-guanylhyazone, MGBG), mitomycin, mitotane (o. p'-DDD), mitoxantrone HCl, octreotide, pentostatin, plicamycin, procarbazine HCl, semustine (methyl-CCNU), streptozocin, tamoxifen citrate, teniposide (VM-26), thioguanine, thiotepa, vinblastine  
25 sulfate, vincristine sulfate, vindesine sulfate, Herceptin, and MabThera. As set forth above and demonstrated by the results of Example X, TPI 792-33 or TPI 792-35 can be administered in conjunction with VP-16 to treat cancer. Similarly, as demonstrated by the  
30 results of Example XIV, TPI 1396-34 also can be administered in conjunction with an anti-cancer drug to treat cancer. Those skilled in the art will appreciate

that similar effects are expected for any active polyphenylurea compound of the invention.

Similarly, for treating pathological conditions which include infectious disease, the methods of the invention can be practiced prior to, during, or subsequent to conventional treatments, such as antibiotic administration, against infectious agents or other methods known in the art. Treatment of pathological conditions of autoimmune disorders also can be accomplished by combining the methods of the invention for derepressing an IAP-inhibited caspase with conventional treatments for the particular autoimmune diseases. Conventional treatments include, for example, chemotherapy, steroid therapy, insulin and other growth factor and cytokine therapy, passive immunity, inhibitors of T cell receptor binding and T cell receptor vaccination. The methods of the invention can be administered in conjunction with these or other methods known in the art and at various times prior, during or subsequent to initiation of conventional treatments. For a description of treatments for pathological conditions characterized by aberrant cell growth see, for example, The Merck Manual, Sixteenth Ed, (Berkow, R., Editor) Rahway, N.J., 1992. Furthermore, anti-cancer drugs including, for example, any of those set forth above with regard to combination compositions, can be administered prior to, during, or subsequent to administration of a derepressor of an IAP-inhibited caspase in a method of treatment.

As described above, administration of a formulation of an agent that derepresses an IAP-inhibited caspase can be, for example, simultaneous with or

delivered in alternative administrations with the conventional therapy, including multiple administrations. Simultaneous administration can be, for example, together in the same formulation or in different formulations  
5 delivered at about the same time or immediately in sequence. Alternating administrations can be, for example, delivering an agent of the invention and a conventional therapeutic treatment in temporally separate administrations. /As described previously, the temporally  
10 separate administrations of an agent of the invention and conventional therapy can similarly use different modes of delivery and routes.

A condition characterized by a pathologically reduced level of apoptosis that can be treated using the  
15 agents and methods of the invention include, for example, cancer, hyperplasia, autoimmune disease and restenosis. A growing number of human diseases have been classified as autoimmune and include, for example, rheumatoid arthritis, myasthenia gravis, multiple sclerosis, psoriasis, systemic  
20 lupus erythematosus, autoimmune thyroiditis, Graves' disease, inflammatory bowel disease, autoimmune uveoretinitis, polymyositis and diabetes. Animal models for many conditions characterized by a pathologically reduced level of apoptosis have been developed and can be  
25 employed for predictive assessment of therapeutic treatments employing an agent that derepresses an IAP-inhibited caspase. Moreover, pharmaceutical compositions of a derepressor of IAP-inhibited caspase can be reliably extrapolated for the treatment of these conditions from  
30 such animal models.

Those skilled in the art will know how to determine efficacy or amounts of an agent of the invention to administer based on the results of routine tests in a relevant animal model. The amount of an agent to be administered can be determined in a clinical setting as well based on the response in a treated individual. Modulation of efficacy, will depend on the pathological condition and the extent to which progression of apoptosis is desired for treatment or reduction in the severity of the pathological condition. Modulation can be accomplished by adjusting the particular agent used to derepress an IAP-inhibited caspase, formulation, or dosing strategy. Based on the guidance provided herein, those skilled in the art will be able to modulate efficacy in response to well known indicators of the severity of the particular condition being treated. For a description of indicators for the various pathological conditions described herein or otherwise known to be characterized by a pathologically reduced level of apoptosis see, for example, The Merck Manual, Sixteenth Ed, (Berkow, R., Editor) Rahway, N.J., 1992.

The following examples are intended to illustrate but not limit the present invention.

25

## EXAMPLE I

Identification of Derepressors of an IAP-Inhibited Caspase  
from Hexapeptide Libraries

This example demonstrates an IAP derepression assay. This Example further demonstrates a positional-

scanning approach to identifying agents that are capable of derepressing an IAP-inhibited caspase.

The DCR390 library consisting of 120 mixtures of hexapeptides was synthesized using methods known in the art as described in R. Houghten *et al.* PCT/US91/08694 and U.S. Patent 5,556,762. Each mixture was made up of a population of hexapeptides all of which had the same amino acid at a defined position and any combination of the 20 essential amino acids at the remaining 5 positions. Each mixture is identified by the position number where the defined amino acid occurs (numbered from 1 to 6 going from the amino-terminus to carboxy-terminus of the hexapeptide) and the identity of the defined amino acid. Thus, as shown in the first column of Table I, the mixture having a tryptophan at position 1 and all combinations of the 20 amino acids at positions 2 through 5 is identified as "position 1, W."

The DCR390 library was screened using the assay set forth below. Based on the mixtures identified from the DCR390 library screen as being capable of derepressing an IAP-inhibited caspase, additional defined positions were incorporated into the TPI 1239 and TPI 1328 sublibraries, and the sublibraries screened using the same assay.

Caspase activity was assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) from Ac-DEVD-AFC synthetic peptides using a Molecular Devices Spectromax 340 (see Zhou *et al.*, J. Biol. Chem. 272:7797-7800 (1997)). Candidate mixtures were screened for the ability to derepress an IAP-inhibited caspase by measuring AFC

hydrolysis rates for mixtures containing purified recombinant caspase-3, Ac-DEVD-AFC, and GST-XIAP in the presence and absence of the candidate agent. The ratio of  $V_{\max}$  for hydrolysis of Ac-DEVD-AFC in the presence and  
5 absence of the candidate mixture was calculated and used to identify those that contain an agent that derepresses an IAP-inhibited caspase. The ratio = ( $V_{\max}$  when candidate mixture, caspase 3 and XIAP are present) / ( $V_{\max}$  when caspase 3 and XIAP are present).

10                Screening of each mixture from the DCR390, TPI 1239 or TPI 1328 library, respectively, using the above described assay was carried out as follows. Each mixture was aliquoted in a 25 microliter volume and in duplicate to a well of a 96 well microtiter plate. Into the first  
15 set of duplicate wells was added 25 microliters of caspase assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10% sucrose, 10 mM DTT, 1 mM EDTA and 0.1% CHAPS) and into the second set of wells was added 25 microliters of a stock solution of 40 nM XIAP in caspase assay buffer.  
20 Each microtiter plate also had the following controls: (1) a buffer blank well to which was added 25 microliters of caspase assay buffer and 25 microliters of peptide carrier solvent, (2) an XIAP control well to which was added 25 microliters of peptide carrier solvent and 25 microliters  
25 of a stock solution of 40 nM XIAP in caspase assay buffer, and (3) a SMAC control well to which was added 25 microliters of a stock solution of 40 nM XIAP in caspase assay buffer, and 2.5 microliters of 4  $\mu$ M SMAC peptide (H-Ala-Val-Pro-Ile-Ala-Gln-Lys-NH<sub>2</sub>, SEQ ID NO:5). Into each  
30 of the sample and control wells was added 25 microliters of 0.64 nM caspase-3 solution followed by 25 microliters of 0.4  $\mu$ M Ac-DEVD-AFC substrate. Fluorescence of



liberated AFC was immediately detected from each well for 30 minutes at 30 second intervals. The  $V_{\max}$  for hydrolysis of AC-DEVD-AFC from each well was measured using the softmax software package.

5                   Results of the screen for the DCR390 library are shown in Table I. Sets of mixtures having the same position fixed with different respective amino acids are arranged in 6 sections identified with the position number. Within each of the six sections are 3 columns  
10 showing (1) the identity of the fixed amino acid, (2) the apparent velocity of the reaction when candidate mixture, caspase 3 and XIAP are present, and (3) the apparent velocity of the reaction when candidate mixture, and caspase 3 are present. Also shown in each section are  
15 results for the XIAP control reaction. The mixtures in each section are arranged in descending order according to apparent velocity in the second column. Those mixtures having significantly higher apparent velocities compared to the XIAP control reaction are listed above the  
20 horizontal line and are thereby identified as containing an agent capable of derepressing an XIAP-inhibited caspase.

TABLE I: DCR 390

Position 1			
	Amino Acid	XIAP+Mixtures	Mixtures
5	W	43	63
	A	35	67
	Y	35	63
	F	34	61
	C	30	62
	L	26	63
10	I	23	64
	E	22	64
	T	22	62
	V	20	60
	M	19	65
	G	17	65
15	P	17	64
	Q	17	66
	XIAP	16	64
	R	16	65
	XIAP	15	64
	H	15	63
20	K	15	65
	S	15	65
	D	14	67
	N	13	63
	XIAPX1.5	24	

Position 2			
	Amino Acid	XIAP+Mixtures	Mixtures
5	W	57	58
	F	44	58
	L	38	61
	C	36	64
	I	32	61
	V	30	59
	Y	30	61
10	A	20	62
	D	20	67
	P	20	60
	R	20	58
	XIAP	19	63
	XIAP	19	63
	G	19	58
15	H	19	58
	M	19	60
	E	18	67
	N	18	64
	T	18	59
	Q	17	59
	S	17	59
20	K	16	61
	XIAPX1.5	29	
25			

Position 3			
	Amino Acid	XIAP+Mixtures	Mixtures
5	F	50	64
	I	36	65
	W	34	65
	L	26	65
	C	20	64
10	V	19	62
	A	16	67
	H	16	70
	K	15	67
	Y	14	63
15	XIAP	13	64
	XIAP	13	64
	D	13	66
	T	13	62
	M	12	67
20	N	12	64
	R	12	67
	E	11	63
	G	11	63
	P	11	67
25	S	11	68
	Q	10	63
	XIAPX1.5	20	

Position 4			
	Amino Acid	XIAP+Mixtures	Mixtures
5	W	52	63
	F	37	61
	L	23	61
10	Y	17	64
	C	15	60
	I	15	60
	V	13	63
	XIAP	12	61
	M	12	64
	N	12	63
15	A	11	64
	XIAP	10	61
	D	10	63
	E	10	59
	G	10	60
	H	10	59
	P	10	66
20	Q	10	64
	S	10	62
	K	9	60
	R	9	62
	T	9	62
	XIAPX1.5	18	
25			

Position 5			
	Amino Acid	XIAP+Mixtures	Mixtures
	W	44	56
	Y	23	57
5	V	15	57
	I	14	57
	L	14	52
	A	13	57
	C	12	54
10	F	12	53
	XIAP	11	52
	XIAP	11	52
	H	11	57
	K	11	55
15	S	11	57
	T	11	56
	D	10	54
	E	10	53
	M	10	49
20	P	10	54
	G	9	57
	N	9	52
	Q	9	54
	R	9	52
25	XIAP1.5	17	

Position 6			
Amino Acid		XIAP+Mixtures	Mixtures
	W	23	51
	R	22	52
5	A	13	53
	C	12	51
	G	12	51
	K	12	53
	Q	11	50
10	XIAP	10	51
	XIAP	10	51
	S	10	53
	Y	10	51
	D	9	50
15	E	9	49
	L	9	53
	M	9	51
	N	9	50
	T	9	53
20	V	9	51
	F	8	50
	H	8	51
	I	8	53
	P	8	49
25	XIAPX1.5	15	

Based on the results of the DCR390 screen, the TPI 1239 library was synthesized and screened using the above-described caspase assay. In particular mixtures were synthesized having positions 5 and 6 defined as tryptophan, positions 3 and/or 4 defined variously, and the remaining positions randomized with the 20 essential

amino acids as set forth in Table II. As shown in Table II, in the absence of XIAP the mixtures had an insignificant effect on caspase activity. Mixtures having ratios of 1.9 or higher in the presence of XIAP were  
5 identified as containing an agent capable of derepressing an IAP-inhibited caspase.



TABLE II: TPI 1239

	Mixture	$V_{\max}(\text{mix+casp3})$	$V_{\max}(\text{mix+casp3+XIAP})$
		$V_{\max}(\text{casp3})$	$V_{\max}(\text{casp3+XIAP})$
5	caspase 3	$1.0 \pm 0.0$	$5.1 \pm 2.3$
	Xiap+caspase3	$0.2 \pm 0.1$	$1.0 \pm 0.0$
	SMAC	$0.8 \pm 0.0$	$3.8 \pm 1.7$
	XXFWWW SEQ ID NO: 11	$0.9 \pm 0.0$	$0.8 \pm 0.1$
	XXLWWW SEQ ID NO: 12	$0.9 \pm 0.0$	$0.7 \pm 0.1$
10	XXWLWW SEQ ID NO: 13	$0.9 \pm 0.0$	$0.7 \pm 0.1$
	XXWWWW SEQ ID NO: 14	$0.9 \pm 0.0$	$0.8 \pm 0.1$
	XXXTWW	$0.8 \pm 0.0$	$4.2 \pm 1.6$
	XXXAWW	$0.9 \pm 0.0$	$3.7 \pm 1.6$
	XXXSWW	$0.8 \pm 0.0$	$3.4 \pm 1.1$
15	XXXQWW	$0.8 \pm 0.0$	$2.4 \pm 0.4$
	XXXKWW	$0.9 \pm 0.0$	$2.3 \pm 1.0$
	XXXVWW	$0.9 \pm 0.0$	$2.2 \pm 0.5$
	XXXRWW	$0.9 \pm 0.0$	$2.1 \pm 0.2$
	XXXHWW	$0.9 \pm 0.0$	$2.1 \pm 0.6$
20	XXXNWW	$0.9 \pm 0.0$	$1.9 \pm 0.6$
	XXXPWW	$0.9 \pm 0.0$	$1.5 \pm 0.2$
	XXXVWW	$0.9 \pm 0.0$	$1.2 \pm 0.3$
	XXXDWW	$0.9 \pm 0.0$	$1.1 \pm 0.2$
	XXXIWW	$0.9 \pm 0.0$	$0.9 \pm 0.1$
25	XXXLWW	$0.9 \pm 0.0$	$0.9 \pm 0.1$
	XXXCWW	$0.9 \pm 0.0$	$0.8 \pm 0.2$
	XXXEWW	$0.9 \pm 0.0$	$0.8 \pm 0.2$
	XXXGWW	$0.9 \pm 0.0$	$0.7 \pm 0.1$
	XXXMWW	$0.9 \pm 0.0$	$0.6 \pm 0.2$
30	XXXFWW	$0.9 \pm 0.0$	$0.6 \pm 0.1$
	XXXWWW	$0.8 \pm 0.0$	not determined
	XXXXWW	$1.0 \pm 0.0$	$2.0 \pm 0.3$

The mixtures identified from the TPI 1239 library as containing an agent capable of derepressing an IAP-inhibited caspase were further analyzed for dose response. The dose response data is provided in Figure 5 11 which shows that the mixtures had no effect on caspase activity. The most active mixtures were found to have alanine, lysine or threonine at position 4, tryptophan at positions 5 and 6 and mixtures at positions 1 through 3.

Based on the results of the DCR390 and TPI 1239 10 library screens, the TPI 1328 library was synthesized and screened using the above-described caspase assay. For each mixture in the TPI 1328 library, 3 to 4 positions were defined and the remaining positions were combinatorialized with all 20 of the essential amino acids 15 including Ala, Asp, Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val, Trp, Cys or Tyr. Position 4 was defined with Ala, Lys or a mixture of Ala, Lys and Thr (the mixture is referred to as "3X" or "A,K,T").

20 Various TPI 1328 sublibraries that were screened and values obtained for the ratio of  $V_{\max}$  for hydrolysis of Ac-DEVD-AFC in the presence and absence of each mixture are plotted in Figure 1. In Figure 1 and Table III, "X" represents a mixture of all 20 essential amino acids and 25 "O" represents the location of the defined position, the identity of the amino acid at the defined position being plotted on the x axis. Candidates having a ratio over 1.7 were identified as being derepressors of XIAP-inhibited caspase-3. A list of derepressors of XIAP-inhibited 30 caspase-3 identified from the TPI 1328 library is provided in Table III.

Table III

	Pos 1	Pos 2	Pos 3	Pos 4	Pos 5	Pos 6	SEQ ID NO:
	X	X	Ala	Ala	Trp	Trp	7
	X	X	Gly	Ala	Trp	Trp	8
5	X	X	Arg	Ala	Trp	Trp	9
	X	X	X	Ala	Trp	Trp	
	X	X	Cys	Lys	Trp	Trp	10
	X	X	Leu	Lys	Trp	Trp	15
	X	X	Gly	3X	Trp	Trp	
10	X	X	Arg	3X	Trp	Trp	
	X	X	Thr	3X	Trp	Trp	
	X	X	Val	3X	Trp	Trp	
	X	Thr	X	3X	Trp	Trp	
	X	Tyr	X	3X	Trp	Trp	
15	Ala	X	X	3X	Trp	Trp	
	Cys	X	X	3X	Trp	Trp	
	Phe	X	X	3X	Trp	Trp	
	Lys	X	X	3X	Trp	Trp	

## Example II

20 Identification of Derepressors of an IAP-Inhibited Caspase  
from the TPI 1332 and TPI 1352 Individual Tetrapeptide  
Libraries

This Example demonstrates identification of agents from the TPI 1332 and TPI 1352 tetrapeptide  
25 libraries that are capable of derepressing an XIAP-inhibited caspase-3.

The TPI 1332 and TPI 1352 tetrapeptide libraries were synthesized identically with the exception that the formyl protecting groups on tryptophan were removed by different procedures. The deprotection step used for the  
5 TPI 1332 library was less complete leaving the possibility that some of the tryptophan residues present on candidate compounds used in the screen retained formyl protecting groups. The deprotecting chemistry used for the TPI 1352 library was substantially complete, however resulted in  
10 the formation of polymeric structures for a subset of the species in the library.

Candidates from the TPI 1332 and TPI 1352 libraries were screened using the derepression assay described in Example I. The ratios of  $V_{\max}$  for hydrolysis  
15 of Ac-DEVD-AFC in the presence and absence of each species of the TPI 1332 and TPI 1352 libraries were determined and those having values greater than 2.4 were identified as derepressors of an IAP-inhibited caspase.

A list of derepressors of XIAP-inhibited  
20 caspase-3 identified from the TPI 1332 and TPI 1352 tetrapeptide libraries is provided in Table IV. Agents identified in both libraries are indicted as "1332/1352."

Table IV

	Agent	Position	Position	Position	Position
		1	2	3	4
5	1332/1352-1	L-Ala	L-Trp	L-Trp	L-ThiAla
	1332/1352-2	L-Ala	L-Trp	L-Trp	L-pClPhe
	1332/1352-47	L-Ala	D-Trp	L-Trp	L-ThiAla
	1332-13	L-Ala	D-Nal	L-Trp	L-Nal
	1332-24	D-Trp	D-Trp	L-Trp	D-Nal
10	1332-41	L-Cha	D-Nal	L-Trp	L-ThiAla
	1352-5	L-Ala	L-Trp	L-Trp	L-3I-Tyr
	1352-6	L-Ala	D-Trp	L-Trp	L-ThiAla
	1352-32	L-Cha	L-Trp	L-Trp	L-pClPhe
	1352-46	L-Ala	D-Trp	L-Trp	D-Trp
15	1352-48	L-Ala	D-Trp	D-Phe	D-Trp
	1352-64	L-Nal	D-Trp	D-Phe	D-Trp
	1352-66	L-Nal	D-Cha	L-Trp	D-Trp
	1352-72	L-Nal	D-ThiAla	D-Phe	D-Trp

Structures of TPI 1332 library compounds are shown in Figure 36A; structure of related compounds of the TPI 1495 series are shown in Figure 37.

20

## Example III

Identification of Individual Peptide Derepressors of an  
IAP-Inhibited Caspase from the TPI 792 Library

This Example demonstrates identification of agents from the TPI 792 library that are capable of  
25 derepressing an XIAP-inhibited caspase-3.

The TPI 792 library is based on a tetrapeptide backbone. The species of the TPI 792 library were

screened in the derepression assay described in Example I. A list of derepressors of XIAP-inhibited caspase-3 identified from the TPI 792 library is provided in Table V. Structures for the TPI 792 core peptides that were  
5 tested are shown in Figure 20.

Table V

Agent	Pos 1	Pos 2	Pos 3	Pos 4	LC mg/ml
792-3	D-Nal	Lys- $\epsilon$ Fmoc	L-pClPhe	Lys- $\epsilon$ Fmoc	2
792-9	D-Nal	D-pClPhe	L-pClPhe	Lys- $\epsilon$ Fmoc	10
10 792-15	D-Nal	L-Nal	L-pClPhe	D-Lys- $\epsilon$ Fmoc	2
792-17	D-Nal	L-Nal	D-Lys (Fm)	Lys- $\epsilon$ Fmoc	2
792-19	L-ThiAla	Lys- $\epsilon$ Fmoc	D-Nal	Lys- $\epsilon$ Fmoc	2
792-22	L-ThiAla	Lys- $\epsilon$ Fmoc	L-pClPhe	D-pFPhe	2
792-27	L-ThiAla	D-pClPhe	L-pClPhe	Lys- $\epsilon$ Fmoc	2
15 792-33	L-ThiAla	L-Nal	L-pClPhe	Lys- $\epsilon$ Fmoc	0.4
792-35	L-ThiAla	L-Nal	D-Lys (Fm)	D-Lys- $\epsilon$ Fmoc	2

The dose response of the agents identified from the TPI 792 library were determined by repeating the derepression assay with variable concentrations of the  
20 agent. Four concentrations were chosen: 0.4, 2, 10 and 50 micrograms per milliliter. From this data the Lowest concentration with a ratio of 2 or higher in the derepression assay (LC) was determined and shown in Table V. The lowest LC value determined from the TPI 792  
25 library was 0.4 micrograms per milliliter for TP1792-33.

## Example IV

Identification of Derepressors of an IAP-Inhibited Caspase  
from the TPI 1313 Library

This Example demonstrates identification of  
 5 agents from the TPI 1313 library that are capable of  
 derepressing an XIAP-inhibited caspase-3.

The TPI 1313 library is based on a tetrapeptide  
 backbone. The species of the TPI 1313 library, listed in  
 Figure 2 and shown in Figure 3, were screened in the  
 10 derepression assay described in Example I. A list of  
 derepressors of XIAP-inhibited caspase-3 identified from  
 the TPI 1313 library is provided in Table VI.

Table VI

Agent	Pos 1	Pos 2	Pos 3	Pos 4
15 1313-4	L-ThiAla	D-pCL-Phe	D-Nal	D-pCL-Phe
1313-5	L-ThiAla	D-pCL-Phe	D-Nal	D-pNO <sub>2</sub> Phe
1313-7	L-ThiAla	D-OEt-Tyr	D-OEt-Tyr	D-pCL-Phe
1313-40	Phe	D-pCL-Phe	D-Nal	D-pCL-Phe

The dose response of the agents identified from  
 20 the TPI 1313 library were determined by repeating the  
 derepression assay with variable concentrations of the  
 agent. Four concentrations were chosen: 0.4, 2, 10 and 50  
 micrograms per milliliter. From this data the apparent  
 IC<sub>50</sub> was determined. The lowest IC<sub>50</sub> value determined from  
 25 the TPI 1313 library range from 3.9 to 6.3 micrograms per  
 milliliter for TPI 1313-7.

## Example V

Identification of Derepressors of an IAP-Inhibited Caspase  
from the TPI 1325 Library

This Example demonstrates identification of  
5 agents from the TPI 1325 library that are capable of  
derepressing an XIAP-inhibited caspase-3.

The TPI 1325 library was screened in the  
derepression assay described in Example I. For each  
species aliquoted in the assay 1 position was fixed (i.e.  
10 having a single known amino acid R group) and three  
positions were combinatorialized. Thus, each "mixture"  
identified from the TPI 1325 library represents a mixture  
of compounds where  $X_1$  and  $X_2$  are selected from Ala, Asp,  
Glu, Phe, Gly, His, Ile, Lys, Leu, Met, Asn, Pro, Gln,  
15 Arg, Ser, Thr, Val, Trp or Tyr and  $X_3$  includes any one of  
Ala, Lys and Thr.

A list of derepressors of XIAP-inhibited  
caspase-3 identified from the TPI 1325 library is provided  
in Table VII.



Table VII

Mixture	Pos 1	Pos 2	Pos 3	Pos 4
1325-10	L-Ala	L-Met	X <sub>1</sub>	X <sub>2</sub>
1325-15	L-Ala	L-Ser	X <sub>1</sub>	X <sub>2</sub>
1325-16	L-Ala	L-Thr	X <sub>1</sub>	X <sub>2</sub>
1325-18	L-Ala	L-Trp	X <sub>1</sub>	X <sub>2</sub>
1325-44	L-Ala	L-ThiAla	X <sub>1</sub>	X <sub>2</sub>
1325-61	L-Ala	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>
1325-64	X <sub>1</sub>	X <sub>2</sub>	L-Trp	D-Trp

10

The dose response of the agents identified from the TPI 1325 library were determined by repeating the derepression assay with variable concentrations of the agent. Four concentrations were chosen: 0.4, 2, 10 and 50 micrograms per milliliter. From this data the apparent IC<sub>50</sub> was determined. The lowest IC<sub>50</sub> value determined from the TPI 1325 library was 12 micrograms per milliliter for TPI 1325-15.

## EXAMPLE VI

20 Identification of Derepressors of an IAP-Inhibited Caspase from the TPI 914, TPI 927, TPI 759 and TPI 882 Libraries

This Example demonstrates identification of agents, from non-peptide based libraries, that are capable of derepressing an XIAP-inhibited caspase-3.

25 The TPI 914, TPI 927, TPI 759 and TPI 882 libraries were screened using positional scanning (as described in U.S. Pat. No. 5,556,762) in combination with the derepression assay described in Example I.

Analysis was started with combinatorial libraries in which at least one position was defined. Hits were identified as those mixtures producing a mixture/XIAP ratio that was greater than or equal to 2. Following  
5 analysis of the first library, libraries of increasing definition were screened until a discrete library was prepared in which all positions were defined. Hits from this defined library were then checked for a dose response which yielded the IC50 values listed below.

10           The TPI 914 N-acyltriamine library included 50 amino acid R groups at position R1, 50 amino acid R groups at position R2 and 50 acid derivatives at position R3 for a total diversity of 125,000 species. Mixtures having a defined functionality at one of the R positions and  
15 identified by positional scanning of the TPI 914 library as having a peptide/XIAP ratio greater than or equal to about 1.8 when present at 25 micrograms per milliliter in the derepression assay were identified and are shown in Figure 4. Control agents having a peptide/XIAP ratio  
20 greater than or equal to about 1.8 when present at 6.25 micrograms per milliliter or 12.5 micrograms per milliliter in the derepression assay were identified and are shown in Figure 5. Additional compounds designed based on this screening are shown in Figure 21A as TPI  
25 1349-1 through TPI 1349-34. The activity of these compounds is shown in Figures 21B-D.

          The TPI 927 polyphenylurea library included 48 amino acid R groups at position R1, 48 amino acid R groups at position R2 and 39 acid derivatives at position R3 for  
30 a total diversity of 89,856 species. Mixtures having a defined functionality at one of the R positions and

identified by positional scanning of the TPI 927 library as having a peptide/XIAP ratio greater than or equal to about 1.8 when present at 4 micrograms per milliliter in the derepression assay were identified and are shown in Figure 6. Control agents having a peptide/XIAP ratio greater than or equal to about 1.8 when present at 25 micrograms per milliliter in the derepression assay were identified and are shown in Figure 9.

10 In particular, as shown in Figure 25a, aliquots of the mixture-based combinatorial library of poly-phenylureas based on TPI 927 described above were added to microtiter plates containing XIAP and caspase-3 (black bars) or, as a control, caspase-3 alone (gray  
15 bars). Caspase-3 activity was measured by monitoring cleavage of the fluorogenic substrate Ac-DEVD-AFC as described herein. Briefly, recombinant proteins were produced in bacteria and purified as described, for example, in Deveraux et al., Nature 388:300-304(1997).  
20 GST-XIAP (46 nM) was added to active caspase-3-His6 (0.36 nM) in 100  $\mu$ l of 50 mM Hepes pH 7.4, 10% sucrose, 1 mM EDTA, 0.1% CHAPS, 100 mM NaCl, and 10 mM DTT to achieve approximately 75% inhibition of protease activity. Activity of caspase-3 was measured by monitoring cleavage  
25 of the fluorogenic tetrapeptide substrate acetyl-DEVD-AFC (BIOMOL, Plymouth, PA) at 100  $\mu$ M. Generation of fluorogenic AFC (7-amino-4-trifluoromethyl coumarin) product was measured with a spectrofluorometric plate reader in kinetic mode for 30 minutes at 37°C using  
30 excitation and emission wavelengths of 405 nm and 510 nm, respectively. Chemical compounds were screened at 6.25, 12.5 and 25.0  $\mu$ g/ml to identify compounds that increase caspase-3 induced cleavage of Ac-DEVD-AFC. Control

reactions lacked XIAP, and all assays were conducted in the linear range of substrate hydrolysis to avoid substrate depletion artifacts.

A representative screen of the positional  
5 scanning combinatorial library (final concentration  
25 mg/ml) is shown in Figure 25a. In Figure 25a, hits  
were defined as compounds that increased caspase-3  
activity greater than or equal to 2 fold in XIAP-inhibited  
reactions without affecting caspase-3 alone. Caspase  
10 activity is presented as the fold increase in enzyme  
velocity after the addition of the compound. The positive  
compound mixtures were deconvoluted by standard methods  
yielding 36 individual compounds which were screened in  
the same caspase derepression assay as described below.

15 The 36 individual compounds (TPI 1396-1 through  
TPI 1396-36) were synthesized based on deconvolution of  
the polyphenylurea library. The individual compounds  
result from the combination of the defined functionalities  
of the most active mixtures of the positional scanning  
20 combinatorial library. The number of functionalities used  
were 3, 4, and 3 at R1, R2 and R3, respectively. As shown  
in Figure 25b, each of the 36 individual compounds was  
tested at 25 mg/ml using the caspase derepression assay  
for their ability to increase caspase-3 activity in the  
25 presence (black bars) or absence (gray bars) of XIAP,  
using a 2-fold elevation in the enzyme velocity as the  
cut-off for positivity. Other cut-offs for positivity can  
include, for example, 1.5 fold and higher, or 1.8 fold and  
higher.

The TPI 759 N-benzyl-1,4,5-trisubstituted-2,3-diketopiperazine library included 29 amino acid R groups at position R1, 27 amino acid R groups at position R2 and 40 acid derivatives at position R3 for a total diversity of 31,320 species. Mixtures having a defined functionality at one of the R positions and identified by positional scanning of the TPI 759 library as having a peptide/XIAP ratio greater than or equal to about 2.0 (or in the case of the sublibrary where R3 was fixed, a ratio of 1.9 or higher) when present at 25 micrograms per milliliter in the derepression assay were identified and are shown in Figure 8. Additional compounds designed based on these functionalities are shown in Figure 23A as TPI 1391-1 through TPI 1391-36. The activity of these compounds is shown in Figures 23B-F.

The TPI 882 C-6-acylamino bicyclic guanidine library included 43 amino acid R groups at position R1, 41 acid derivatives at R2 and 41 acid derivatives at R3 for a total diversity of 72,283 species. Mixtures having a defined functionality at one of the R positions and identified by positional scanning of the TPI 882 library as having a peptide/XIAP ratio greater than or equal to about 1.9 when present at 5 micrograms per milliliter in the derepression assay were identified and are shown in Figure 7. Control agents having a peptide/XIAP ratio greater than or equal to about 2.0 when present at 8 micrograms per milliliter in the derepression assay were identified and are shown in Figure 10. Additional compounds designed based on these functionalities are shown in Figure 24A as TPI 1400-1 through TPI 1400-58. The activity of these compounds is shown in Figures 24B-H.

## EXAMPLE VII

SMAC Competition Assay

This Example describes an assay useful for determining the binding affinity of a derepressor of an IAP-inhibited caspase for an IAP, or functional fragment thereof.

A polarization based binding assay was used to detect binding between rhodamine labeled SMAC (rhodamine-SMAC) and the XIAP fragments BIR2 or BIR3RING. The assay is based on the decrease in mobility that occurs for rhodamine-SMAC when associated with XIAP or functional fragments thereof which is detected as a reduction in polarization for bound rhodamine-SMAC compared to free (unbound) rhodamine-SMAC.

Binding affinity of rhodamine-SMAC for a glutathione-S-transferase-BIR2 fusion protein (GST-BIR2) or BIR3RING was determined as follows. A 1 milliliter reaction cocktail was made to include 100 mM  $\text{NaPO}_4$ , 0.01% bovine gamma globulin, and 20 nM rhodamine-SMAC. 0.1 ml aliquots taken from the reaction cocktail were placed into the 8 wells of a column in 96 well assay plate. Twelve such columns were set up each with a different concentration of GST-BIR2 ranging from 0 to 495 nM. Polarization of Rhodamine in each well was read in an LJJL Analyst HT with excitation at 530 nm and emission at 580 nm. Data was averaged for 8 samples having the same concentration of GST-BIR2 then plotted as a function of millipolars vs. the concentration of GST-BIR2. The same procedure was followed for BIR3RING. Rhodamine-SMAC at 20

nM had a  $K_d$  for GST-BIR2 of 100 nM and a  $K_d$  for BIR3RING of 80 nM.

The polarization assay was used to identify agents having the ability to compete with SMAC for binding to XIAP.

Unlabelled SMAC was titrated against a solution containing 20 nM rhodamine-SMAC and 225 nM GST-BIR2. Unlabeled SMAC was titrated in the range of 0 to 20  $\mu$ M in 4  $\mu$ M increments. Again 8 samples (0.1 ml) were counted for each concentration of unlabeled SMAC allowing for the determination of a standard error. Data was plotted as a function of millipolars vs SMAC and  $IC_{50}$  values determined. The  $IC_{50}$  value of the SMAC titration was 12 mM. Identical procedures were followed for titration of unlabeled SMAC against a rhodamine-SMAC and BIR3RING containing solution. In this case, the  $IC_{50}$  value of SMAC was 8 mM.

Candidate agents from a library are added to a solution containing 20 nM rhodamine-SMAC and 225 nM GST-BIR2. Fluorescence polarization is determined for each sample and those candidates that show a decrease in polarization compared to a control reaction containing 20 nM rhodamine-SMAC and 225 nM GST-BIR2 are identified as derepressors of an IAP-inhibited caspase. As a control, fluorescence polarization is also determined for the library sample in the absence of GST-BIR2.

An agent identified as a derepressor of an IAP-inhibited caspase is titrated against a solution of rhodamine-SMAC and GST-BIR2. Polarization is determined

at each concentration of the agent as described above. Data is plotted as a function of millipolars vs. SMAC and binding constants determined also as described above.

#### EXAMPLE VIII

##### 5    Screening of Individual Compounds from Various Libraries

This example describes screening of individual agents derived from TPI 914, TPI 927, TPI 759 and TPI 882 libraries and identification of individual agents that Derepress an IAP-Inhibited Caspase.

10

Individual agents were synthesized based on the active agents identified in Example VI. Selected agents based on the TPI 914 derepressors shown in Figure 5 were synthesized and are identified as agents TPI 1349-1  
15 through TPI 1349-34 in Figure 21. Selected agents based on the TPI 927 derepressors shown in Figure 9 were synthesized and are identified as agents TPI 1396-1 through TPI 1396-65 in Figure 22. Selected agents based on the TPI 759 derepressors shown in Figure 8 were  
20 synthesized and are identified as agents TPI 1391-1 through TPI 1391-36 in Figure 23. Selected agents based on the TPI 882 derepressors shown in Figure 10 were synthesized and are identified as agents TPI 1400-1 through TPI 1400-58 in Figure 24.

25

The caspase derepression assay was used to evaluate the agents shown in Figures 21-24. Each compound was tested using the caspase derepression assay for its ability to increase caspase-3 activity. The structures



for TPI 1349-1 through TPI 1349-34 along with respective molecular weights and masses are shown in Figure 21A. The activity of TPI 1349-1 through TPI 1349-34 in a caspase derepression assay using full length XIAP is shown in Figure 21B. The activity of TPI 1349-1, -3, -8, -13, -23, and -28 using both full length XIAP and XIAP BIR2 domain is shown in Figure 21C. The activity of TPI 1349-1, -3, -8, -13, -23, and -28 using cIAP-1 BIR2 domain is shown in Figure 21D. These data indicate that the TPI 1349 compounds are active in derepressing caspase inhibited by either XIAP or the BIR2 domain of XIAP, but do not overcome cIAP1-mediated suppression of caspase-3. It is important to note that the lack of activity observed for various compounds can be the result of the compounds being present at a two fold excess over cIAP1.

The structures of TPI 1396-1 through TPI 1396-65 along with respective molecular weights and masses are shown in Figure 22A. The activity of TPI 1396-1 through TPI 1396-36 in a caspase derepression assay using full length XIAP is shown in Figure 22B. The activity of TPI 1396-37 through TPI 1396-65 in the derepression assay using full length XIAP is shown in Figure 22C. A table indicating the activities of TPI 1396-11, -12, -22, -28, and -34 in the derepression assay using full length XIAP and the XIAP BIR2 domain is shown in Figure 22D. The activity of TPI 1396-11, -12, -22, -28, and -34 at 50  $\mu\text{g/ml}$  using XIAP BIR2 domain is shown in Figure 22E. Additional representative data for the activity of TPI 1396-11, -12, -22, -28, and -34 at 100  $\mu\text{g/ml}$  using full length XIAP and Caspase 3 or 7 is shown in Figure 22F. The activity of TPI 1396-11, -12, -22, -28, and -34 at 100  $\mu\text{g/ml}$  using cIAP-1 BIR2 domain is shown in Figure 22G.

These data indicate that TPI 1396 compounds are active in derepressing caspase inhibited by either XIAP or the BIR2 domain of XIAP, but do not overcome cIAP1-mediated suppression of caspase-3. It is important to note that the lack of activity observed for various compounds can be the result of the compounds being present at a two fold excess over cIAP1.

The structures of TPI 1391-1 through TPI 1391-36 along with respective molecular weights and masses are shown in Figure 23A. The activity of TPI 1391-1 through TPI 1391-36 at 100 µg/ml in a caspase derepression assay using full length XIAP is shown in Figure 23B. The activity of TPI 1391-1 through TPI 1391-36 at 25 µg/ml in the derepression assay using full length XIAP is shown in Figure 23C. A table indicating the activities of TPI 1391-1, -4, -5, 7, -17, -21, -25, -28, -34 and -35 in the derepression assay using full length XIAP is shown in Figure 23D. A comparison of the activities of TPI 1391-1, -4, -5, 7, -17, -21, -25, -28, -34 and -35 in the derepression assay using full length XIAP or XIAP BIR2 domain is shown in Figure 23E. The activity of TPI 1391-1, -4, -5, 7, -17, -21, -25, -28, -34 and -35 using cIAP1-BIR2 domain is shown in Figure 23F. These data indicate that TPI 1391 compounds are active in derepressing caspase inhibited by either XIAP or the BIR2 domain of XIAP, but do not overcome cIAP1-mediated suppression of caspase-3. It is important to note that the lack of activity observed for various compounds can be the result of the compounds being present at a two fold excess over cIAP1.

The structures of TPI 1400-1 through TPI 1400-58 along with respective molecular weights and masses are

shown in Figure 24A. The activity of TPI 1400-1 through TPI 1400-28 at 25 µg/ml in a caspase derepression assay using full length XIAP is shown in Figure 24B. The activity of TPI 1400-1 through TPI 1400-28 at 10 µg/ml in the derepression assay using full length XIAP is shown in Figure 24C. The activity of TPI 1400-29 through TPI 1400-58 at 25 µg/ml in the derepression assay using full length XIAP is shown in Figure 24D. The activity of TPI 1400-29 through TPI 1400-58 at 10 µg/ml in the derepression assay using full length XIAP is shown in Figure 24E. A table indicating the activities of TPI 1400-6, -7, 13, -14, -33, -37, -43, -44 in the derepression assay using full length XIAP is shown in Figure 24F. A comparison of the activities of TPI 1400-6, -7, 13, -14, -33, -37, -43, -44 in the derepression assay using full length XIAP or XIAP BIR2 domain is shown in Figure 24G. The activity of TPI 1400-6, -7, 13, -14, -33, -37, -43, -44 using cIAP BIR2 domain is shown in Figure 24H. These data indicate that TPI 1400 compounds are active in derepressing caspase inhibited by either XIAP or the BIR2 domain of XIAP, but do not overcome cIAP1-mediated suppression of caspase-3. It is important to note that the lack of activity observed for various compounds can be the result of the compounds being present at a two fold excess over cIAP1.

25

#### EXAMPLE IX

##### Peptidyl and Non-Peptidyl Compounds Restore Caspase Activity of IAP-Inhibited Caspase *in vitro*

30 This example demonstrates an assay for determining potency of peptidyl and non-peptidyl derepressors of IAP-inhibited caspases *in vitro*. This

example also identifies peptidyl and non-peptidyl compounds having potency at restoring caspase activity of IAP-inhibited caspase *in vitro*.

5           The caspase derepression assay was used to evaluate peptidyl IAP antagonists identified from screens of the TPI 792 library and non-peptidyl IAP antagonists identified from screens of the TPI 1391 and TPI 1396 libraries. Each compound was titrated against a solution  
10 of rhodamine labeled SMAC tetrapeptide, AVPI (SEQ ID NO:4), and full length XIAP under the conditions described in Example VII. Polarization was determined at each concentration of the IAP antagonist, data was plotted as a function of millipolars vs. compound concentration, and  
15 the EC50 binding constants were determined from the plots. As a control unlabeled SMAC tetrapeptide, AVPI (SEQ ID NO:4) was also assayed.

For several compounds of the TPI 1396 library (TPI 1396-11, -12, -22, -28, and -34), the caspase  
20 derepression assay was carried out in the presence of caspase-3 or caspase-7. These studies, representative results of which are shown in Figure 22F, polyphenylureas reversed XIAP-mediated suppression of caspases 3 and 7. For these experiments, GST-XIAP was added to active  
25 caspase-3 (0.69 nM) or caspase 7 (3.2 nM) and 75  $\mu$ M compounds with 100  $\mu$ M DEVD-AFC in a 100  $\mu$ l of buffer. Generation of AFC was measured in a spectrofluorimeter with 405 nm excitation and 510 nm emission at 37°C for 30 minutes. The data shown in Figure 22F represent caspase  
30 activity, compared to XIAP-inhibited reactions (=1.0) and are mean  $\pm$  standard deviation of three determinations. As

a control unlabeled SMAC heptapeptide, AVPIAQK was also assayed.

Table VIII summarizes the results of the SMAC competition assay for IAP antagonists identified from the TPI 792, TPI 1391 and TPI 1396 libraries. The EC50 was determined, by calculating the amount of compound necessary to restore caspase-3 activity to 50% of maximum velocity (Vmax). Two of the most potent tetramer peptides were TPI 792-33 and TPI 792-35 which displayed enzyme derepression activities *in vitro* that were 5.2 to 2.5 fold better than SMAC peptide, respectively. The most potent diketopiperazine based compounds included TPI 1391-21, TPI 1391-28 and TPI 1391-34 which exhibited potencies 3.3 to 5.0 fold more active than SMAC peptide. The most potent phenyl-urea compounds included TPI 1396-22, TPI 1396-34 and TPI 1396-28 which exhibited potencies that were 1.6 to 2.8 fold more active than SMAC peptide.

The caspase derepression assay was used to evaluate non-peptidyl IAP antagonists identified from screens of the TPI 1396 library in the presence of the cIAP1 BIR2 domain. Each compound was present at a 100  $\mu$ M with caspase-3 at 8.5 nM, cIAP BIR2 at 37  $\mu$ M and 100  $\mu$ M Ac-DEVD-AFC. Assays were initiated upon addition of DEVD substrate and release of fluorogenic product was followed in the kinetic mode for 30 minutes at 37 °C. Assays were performed in a Molecular Devices FMAX spectrofluorimeter. Ratios are relative to assay with cIAP BIR2 in the absence of compound. Figures 22B and 22C show relative caspase activity in the presence of various TPI 1396 library compounds. As is shown in Figure 22G, polyphenylurea compounds inhibit XIAP but do not inhibit cIAP1, as

assayed using the cIAP1 BIR2 domain. Compounds TPI 1396-11, TPI 1396-12, TPI 1396-22 and TPI 1396-34 represent active XIAP inhibitors, while TPI 1396-28 is an inactive analog.

5

Table VIII

	Natural peptides	EC50 ( $\mu$ M)	Relative Potency
	SMAC AVPI tetrapeptide (SEQ ID NO:4)	125	1.0
	Un-natural peptides		
10	TPI 792-33	24	5.2
	TPI 792-35	51	2.5
	Diketopiperazines		
	TPI 1391-21	33.6	3.7
	TPI 1391-28	25.1	5
	TPI 1391-34	39.4	3.3
	Diphenyl and Triphenyl Ureas		
15	TPI 1396-22	45.3	2.8
	TPI 1396-34	77.1	1.6
	TPI 1396-28	>134	N/A

These results demonstrate that peptidyl  
 20 compounds TPI 792-33 and TPI 792-35; diketopiperazine  
 based compounds TPI 1391-21, TPI 1391-28 and TPI 1391-34;  
 and phenyl-urea compounds TPI 1396-22 and TPI 1396-34  
 derepressed XIAP inhibited caspase *in vitro* and did so

with more potency than the SMAC AVPI tetrapeptide (SEQ ID NO:4).

#### EXAMPLE X

##### 5    Peptidyl Compounds TPI 792-33 and TPI 792-35 Kill Tumor           Cells

          This example demonstrates an assay for  
determining potency of derepresors of IAP-inhibited  
caspases in cell cultures. This example also demonstrates  
10 that TPI 792-33 and TPI 792-35 reduce the viability of  
tumor cells in culture.

          The TPI 792-33 and TPI 792-35 compounds were  
assayed to determine their effects on tumor cell  
viability. As shown in Figure 12, TPI 792-33 and TPI  
15 792-35 are tetrapeptides composed of unnatural amino acids  
that differ in their amino acid sequence at the third  
position. The TPI 792-33 and TPI 792-35 compounds both  
have L-3-(2-thienyl)-alanyl, L-(2-naphthyl)-alanyl, and  
L-(e-fluorenylmethyloxycarbonyl)-lysine moieties at  
20 positions 1 (N-terminus), 2 and 4, respectively, but  
differ at position 3 where TPI 792-33 has  
L-p-chloro-phenylalanyl and TPI 792-35 has a  
D-(e-fluorenylmethyloxycarbonyl)-lysyl moiety.

          Cells from the prostate cancer cell line, ALVA31  
25 express XIAP, as well as other IAP-family proteins. The  
*in vivo* effects of TPI 792-33 or TPI 792-35, either  
individually or in combination with the cytotoxic  
anticancer drug VP-16 (etoposide), on derepression of  
XIAP-inhibited caspase and viability of ALVA31 cells was

determined as follows. ALVA31 prostate cancer cells were seeded onto 96 well plates ( $10^4$  cells/well) in 100  $\mu$ L RPMI containing 2.5% fetal bovine serum (FBS). After 24 hours, the IAP antagonists TPI 792-33, TPI 792-35 or the SMAC  
5 AVPI tetrapeptide (SEQ ID NO:4) was added at a final concentration of 40  $\mu$ M with or without VP-16 (100  $\mu$ M final concentration). After another 24 hrs incubation, cell viability was measured by the XTT dye-reduction assay (Roche, Molecular Biochemicals; Indianapolis, Ind.) and  
10 trypan blue dye exclusion assay.

Anti-cancer drug VP-16 (etoposide), when administered alone to ALVA31 cells, had essentially no effect on the viability of the cells in the XTT dye-reduction assay (Figure 13) and trypan blue dye  
15 exclusion assay. The SMAC AVPI tetrapeptide (SEQ ID NO:4), when administered alone to the ALVA31 cells, also had no effect on cell viability. In contrast, the TPI 792-33 and TPI 792-35 peptides reduced viability of these prostate cancer cells by nearly half. Moreover, the  
20 combination of VP-16 with these peptides resulted in more potent tumor cell killing compared to VP-16 alone. By comparison, the SMAC peptide was inactive, failing to significantly reduce the relative number of viable tumor cells under the same culture conditions.

25               These results demonstrate that TPI 792-33 and TPI 792-35 display markedly improved cellular activity compared to wild-type AVPI peptide from SMAC (SEQ ID NO:4). Furthermore, these results indicate that TPI 792-33 and TPI 792-35 have the effect of increasing  
30 apoptosis in tumor cells by derepressing IAP-inhibited caspase. These results also demonstrate that TPI 792-33



and TPI 792-35 sensitize prostate cancer cells to the anticancer drug VP-16.

#### EXAMPLE XI

##### Non-Peptidyl Compounds TPI 1396-34 and TPI 1391-28 Kill 5 Tumor Cells

This example demonstrates that phenyl urea compounds (also called polyphenylurea compounds) identified from the TPI 1396 library and diketopiperazine  
10 compounds identified from the TPI 1391 library reduce the viability of tumor cells in culture. This example further demonstrates that cell killing activity for TPI 1396-34 and TPI 1391-28 is specific for tumor cells.

The following assay was used to test the  
15 ability of individual compounds from the TPI 1396 and TPI 1391 libraries to induce apoptosis of cultured tumor cell lines. Each of the compounds listed in Table IX was individually added to Jurkat leukemia cells ( $6.25 \times 10^5$  cells/mL) in RPMI containing 2.5% FBS at various  
20 concentrations for 20 hours. After incubation, cells were washed and stained with FITC-conjugated Annexin V antibody and propidium iodide (Biovision; Mountain View, CA). Cells were incubated for 20 minutes at room temperature in the dark and fluorescence was measured by flow cytometry  
25 (FACScan, Immunocytometry system; Becton-Dickinson; San Jose, CA). Cells staining positive for Annexin V were deemed non-viable.

As shown in Table IX, these compounds were able to induce cell death in a concentration dependent manner.

Although SMAC was able to reduce cell viability by about 16% when present at 50  $\mu$ M, several TPI 1396-34 and TPI 1391-28 compounds reduce cell viability by about 85 to 94%. Thus, compounds identified from the TPI 1396-34 and  
5 TPI 1391-28 libraries were about 5 to 6 fold more potent than SMAC at inducing apoptosis in tumor cells. A representative experiment testing additional compounds is shown in Figure 27a.

Table IX

		TPI 1391 %nonviable cells						
Concentration		100	50	25	10	5	5	1
$\mu\text{M}$								
	1391-28		91	87	55	12	28	19
	1391-21		94	91	44	11	18	16
	1391-25		87	90	60	22	49	16
5	1391-17		91	88	45	N.T.	25	13
	1391-5		88	88	36	N.T.		17
	1391-1		91	69	20	N.T.		18
	1391-4		86	90	48	12	18	20
		TPI 1396 %nonviable cells						
Concentration		100	50	25	10	5	5	1
$\mu\text{M}$								
	1396-34		85	83	62	73	51	13
10	1396-12		85	89	89	95	95	15
	1396-11		90	90	90	97	95	14
	1396-28		13	14	13			13
	SMAC	15	16	16		12		

The TPI 1396-34 and TPI 1391-28 compounds were further tested as set forth below. Figure 14 (Panel B) shows the structures for phenyl urea TPI 1396-34 and diketopiperazine TPI 1391-28. Both of these compounds were shown to induce apoptosis of cultured tumor cell lines in a concentration-dependent manner using the assay

described above, except that compounds were added in the range of 0 to 20  $\mu\text{M}$ .

As shown in Figure 15, TPI 1396-34 and TPI 1391-28 killed Jurkat leukemia cells with  $\text{EC}_{50}$  values of about 6.5  $\mu\text{M}$  following a one-day exposure. Control compounds having the same core pharmacophore structure but with different substituents at the R group which prevent binding to XIAP, did not significantly reduce the viability of Jurkat leukemia cells under the assay conditions.

Comparison of cell killing by TPI 1396-34 and TPI 1391-28 to the respective control compounds is shown in Figure 16. One day treatment of Jurkat leukemia cells with 5  $\mu\text{M}$  or 8  $\mu\text{M}$  of TPI 1396-34, killed about 75% and 85% of cells, respectively. One day treatment of Jurkat leukemia cells with 5  $\mu\text{M}$  or 8  $\mu\text{M}$  of TPI 1391-28, killed about 45% and 80% of cells, respectively. In contrast, the control compounds had no significant effect on the viability of these leukemia cells compared to untreated cells, indicating that the cytotoxic activity of these compounds is specific. Under the same assay conditions, 5 or 8  $\mu\text{M}$  of the SMAC AVPI tetrapeptide (SEQ ID NO:4) had no significant effect on the viability of these leukemia cells, confirming that TPI 1396-34 and TPI 1391-28 had far greater potency than SMAC. Figure 27b shows a representative assay as described above where Jurkat cells were cultured with 10  $\mu\text{M}$  1396-34 for various times before measuring percent cell death by annexin-V staining. As can be seen in Figure 27b, the kinetics of apoptosis induction of Jurkat cells by TPI 1396-34 was rapid, with

half-maximal killing achieved at approximately 12 hours and maximum killing at about 24 hours.

Jurkat cells were also cultured with TPI 1396-34, a structurally related compound TPI 1396-28, or SMAC 4-mer peptide AVPI (SEQ ID NO: 4) at final concentrations of 8  $\mu$ M for 20 hours. After incubation, caspase-3 and caspase-7 activity was measured in whole cells using a cell permeable substrate. As expected, TPI 1396-34 induced caspase activation, while TPI 1396-28 (which differs from TPI 1396-34 only at R2) did not induce caspase activation (see Figure 27c).

In addition, Jurkat cells were cultured with various concentrations of TPI 1396-34 with or without 100  $\mu$ M zVAD-fmk, which is a broad spectrum caspase inhibitor. The percentage of cell death was measured 20 hours later by annexin-V staining. Apoptosis induced by TPI 1396-34 was suppressible by co-culturing the cells with zVAD-fmk, (see Figure 27d).

A comparison of the effects of TPI 1396-34 on normal bone marrow cells versus Jurkat leukemia cells was performed as follows. TPI 1396-34 (5  $\mu$ M) was incubated with Jurkat cells or normal bone marrow mononuclear cells ( $6.25 \times 10^5$ /mL) in RPMI and 2.5% FBS for 20 hours. After incubation, cells were washed, stained with FITC-conjugated Annexin V antibody and propidium iodide, and fluorescence measured by flow cytometry as described above.

As shown in Figure 17, TPI 1396-34 and TPI 1391-28 caused little toxicity to normal bone marrow cells

under the same culture conditions where robust killing of the leukemia cells was observed. These results demonstrate that the TPI 1396-34 and TPI 1391-28 selectively kill tumor cells compared to normal cells.

5

## Example XII

Killing of Tumor Cells by TPI 1396-34 is Mediated by XIAP

This Example describes the effects of over-expressing wild type and mutant XIAP on tumor cell killing by TPI 1396-34.

10           U937 leukemia cells ( $6.25 \times 10^5$  cells/mL) that had been stably transfected with either a Neo-control plasmid (U937-Neo cells) or a plasmid encoding XIAP (U937-XIAP cells) were treated with 5 or 8  $\mu$ M of TPI 1396-34 in RPMI and 2.5% FBS for 20 hours. After incubation, cells  
15 were washed, stained with FITC-conjugated Annexin V antibody and propidium iodide, and fluorescence measured by flow cytometry as described in Example XI.

As shown in Figure 18 and Figure 27e, over-expression of XIAP rendered U937 cells resistant to TPI  
20 1396-34. Comparisons of the effects of TPI 1396-34 on U937-Neo and U937-XIAP cells demonstrated that over-expression of XIAP correlated with resistance to apoptosis induction by this agent. The increased resistance of tumor cells to the apoptogenic effects of  
25 TPI 1396-34 when the cells over-express XIAP indicates that TPI 1396-34 induces apoptosis by binding to XIAP.

Over-expression of XIAP in the U937-XIAP cells compared to vector transfected control cells was confirmed by immunoblotting (Figure 18, upper right panel). Expression of XIAP in K562 cells was included as a control, as these cells are known to express XIAP endogenously. Equal amounts of protein were subjected to SDS-PAGE (4-20% gradient gels from ISC BioExpress, Kaysville, UT), followed by transfer to nitrocellulose membranes. Membranes were probed with monoclonal mouse-anti human XIAP (0.25 mg/mL) (Transduction Laboratories, Lexington, KY) or monoclonal mouse-anti b-actin (1:3000 v/v) (Sigma Inc, Milwaukee, WI). Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA). Detection was performed by the enhanced chemiluminescence (ECL) method. In Figure 27e, U937 cells stably over-expressing XIAP or neomycin control transfectants were cultured with various concentrations of TPI 1396-34 for 20 hours and the percentage of cell death was measured by annexin-V staining. Lysates were prepared from the cells, normalized for total protein content and analyzed by SDS-PAGE immunoblotting using antibodies specific for XIAP, caspase-3, and  $\beta$ -actin.

In addition to transfecting the cells with full-length XIAP, analogous assays were performed with HeLa cells transfected with plasmids over-expressing various XIAP mutants. HeLa cells were transiently transfected with plasmids encoding full-length, wild-type XIAP versus deletion mutants having only the BIR2 (caspase-3/7 suppressing) domain, BIR3 (caspase-9 suppressing) domain, or a mutant in which both of the putative SMAC-binding pockets in BIR2 and BIR3 had been

mutated to no longer bind caspases. The mutant was produced by site-directed mutagenesis to modify positions 148, 219, 223, 314 and 323 to contain alanine. HeLa cells were also transiently transfected with plasmids encoding  
5 Bcl-XL, an anti-apoptotic protein that operates upstream of caspases to suppress Cytochrome C release from mitochondria. HeLa cells ( $2.5 \times 10^5$ ) were seeded onto six well plates in 2 mL DMEM H21 with 5% FBS. After 24 hrs, cells were transfected (Gene Porter) with plasmids. At 48  
10 hrs after transfection, cells were treated with 5  $\mu$ M of TPI 1396-34 for 20 hours. Both floating and adherent cells were then recovered from cultures, washed, and apoptosis was determined by Annexin V staining using flow cytometry.

15 As shown in Figure 19, TPI 1396-34 induced apoptosis in HeLa cells transfected with a control vector. Apoptosis induced by TPI 1396-34 was not blocked by over-expressing Bcl-XL, consistent with the fact that Bcl-XL operates upstream of XIAP. In contrast, HeLa cells  
20 over-expressing full-length XIAP were protected from TPI 1396-34. In addition, cells expressing a mutant of XIAP in which the SMAC-binding pocket of XIAP was mutated were not protected from the chemical compound nor were cells expressing a mutant comprised of only the BIR2 domain.  
25 Cells expressing the BIR3 domain were protected from the apoptogenic activity of TPI-1396-34. Taken together, these results indicate that TPI 1396-34 induces apoptosis of tumor cell lines in culture by targeting XIAP.

As shown in Figure 27f, HeLa cells were  
30 transfected with plasmids encoding XIAP, Bcl-X, CrmA, or empty vector. At 2 days after transfection, cells were



treated with TPI 1396-34 (5  $\mu$ M) for 20 hours and the percentage of dead cells was measured by annexin-V staining. Consistent with IAPs representing a target of the polyphenylurea compounds disclosed herein, transient or stable over-expression of XIAP rendered tumor cell lines more resistant to apoptosis induction by active compound, shifting the dose-response curve to the right, so that higher concentrations of compound were required (Figure 27e and 27f). In contrast, over-expressing anti-apoptotic proteins Bcl-XL or CrmA did not alter sensitivity of tumor cell lines to TPI 1396-34, demonstrating a specific effect (see Figure 27f). Bcl-XL over-expression did afford resistance to traditional anticancer drugs such as etoposide and CrmA (a caspase-8 inhibitor), and protected cells from apoptosis induced by TRAIL, confirming that these anti-apoptotic proteins were functional in these experiments.

### EXAMPLE XIII

#### Broad Activity of Polyphenylurea Compounds Against Transformed Cells

This Example shows polyphenylurea compounds have activity against many different tumor cell lines, while having little effect on normal cells.

Selected polyphenylurea compounds were tested on the National Cancer Institute (NCI) panel of 60 tumor cell lines (see Figure 28a and Figure 29). Cells were cultured with compounds for 48 hours followed by measurement of the relative number of viable cells using a protein-based colorimetric assay, expressing data as percent growth

relative to cells treated with solvent control alone.

Compounds TPI 1396-11, TPI 1396-12, TPI 1396-22, and TPI 1396-34 induced reductions in viable cell numbers, with an average LD<sub>50</sub> (concentration required to kill 50% of the cells, after adjustment for background cell death) for the 60 cell lines of 10 +/- 2.8  $\mu$ M (median = 17  $\mu$ M), 7.6 +/- 12  $\mu$ M (median = 6  $\mu$ M), 11 +/- 2.6  $\mu$ M (median = 22  $\mu$ M), and 22 +/- 5  $\mu$ M (median = 23  $\mu$ M), respectively.

Moreover, the LD<sub>50</sub> was <10  $\mu$ M for over one-third of the tumor cells treated with the active compounds. In contrast, LD<sub>50</sub> was not reached for any of the 60 tumor cell lines after treatment with up to 70  $\mu$ M of the structurally related control compound TPI 1396-28 (see Figure 28a). By comparison, when using this same assay, the mean LD<sub>50</sub> for the anticancer drug etoposide in the NCI panel of 60 tumor cell lines is 200 +/- 2.5  $\mu$ M, with none of the cells having LD<sub>50</sub> < 10  $\mu$ M.

Compared to tumor cell lines, normal cells were relatively resistant to the polyphenylurea compounds. As shown in Figure 28b, various types of cells including Jurkat and HeLa tumor cell lines, and normal peripheral blood lymphocytes (PBLs), bone marrow mononuclear cells, mouse embryo fibroblasts (MEFs), or human prostate epithelial cells were cultured with various concentrations of TPI 1396-34. After 2 days, cell viability was assessed by annexin-V staining.

When normal cells such as mouse embryo fibroblasts (MEFs), human prostate epithelial cells, and peripheral blood lymphocytes (PBLs) were cultured with various concentrations of active poly-phenylurea compound TPI 1396-34, the slope of the cell cytotoxicity curve was

much flatter than observed for tumor cell lines (Figure 28b). At a concentration of 10 mM, for example, the percentage cell death increased by less than one-fold above background for these types of normal cells, while  
5 killing of tumor lines such as Jurkat and HeLa increased by > 4 fold. Activating lymphocytes with the mitogenic lectin, phytohemagglutinin (PHA), did not increase sensitivity to the XIAP-antagonists. Normal human bone marrow mononuclear cells (BM) tended to be more sensitive.  
10 However, even for these cells, the LD<sub>50</sub> was not reached at concentrations up to 40 mM. By comparison, the LD<sub>50</sub> of Jurkat and HeLa cells was achieved at concentrations of about 5  $\mu$ M (Figure 28b).

Since many tumor and leukemia cell lines  
15 proliferate faster than normal cells, it was investigated whether the polyphenylurea compounds could induce apoptosis of non-replicating malignant cells. Accordingly, freshly isolated chronic lymphocytic leukemia (CLL) B-cells from five patients and freshly isolated  
20 leukemic blasts from five patients with acute myelogenous leukemia (AML) were treated for 20-24 hours *in vitro* with compounds TPI 1396-11, TPI 1396-12, or TPI 1396-34 versus TPI 1396-28 control compound or AVPI peptide (SEQ ID NO: 4), and the percentage of cell death  
25 was measured by annexin-V/propidium iodide staining with FACS analysis (Figure 28c) or annexin-V staining (Figure 28d). These leukemic cell samples contained only small percentages of cycling cells, and did not replicate under standard culture conditions. As seen in Figures 28c and  
30 28d, active polyphenylurea compounds induced dose-dependent cell death of primary-cultured leukemia cells in 5 of 5 CLL specimens (Figure 28c) and 4 of 5 AML

specimens (Figure 28d) examined, with LD<sub>50</sub> achieved at doses of approximately 5  $\mu$ M after correction for spontaneous apoptosis in culture. In contrast, the inactive control compound TPI 1396-28 and the AVPI peptide  
5 did not induce apoptosis of these leukemia cells. All samples were treated with both TPI 1396-34, TPI 1396-28, as well as the AVPI peptide, but the complete data set is shown only for AML-1. Comparable results were obtained with control compounds for the other leukemia specimens.  
10 In addition, the active polyphenylurea compounds were also active against transformed hematopoietic cells from mice, inducing death of mouse 70Z/3 lymphoma and immortalized 32D myeloid cells with EC<sub>50</sub> values of 8 to 12  $\mu$ M. Thus, cell replication is not required for sensitivity to the  
15 disclosed poly-phenylurea compounds.

#### EXAMPLE XIV

##### Polyphenylurea Compounds Sensitize Tumor Cells to Anticancer Drugs and TRAIL

This example shows that polyphenylurea compounds  
20 can collaborate with conventional anticancer drugs to induce killing of tumor cells.

In order to test the effect of polyphenylurea compounds in combination with known anticancer drugs, Du145 prostate cancer cells were cultured for 48 hours  
25 with various concentrations of Etoposide (VP16), Doxorubicin (DOX), or Paclitaxel (TAXOL) with or without 10  $\mu$ M TPI 1396-34. The percentage of viable cells relative to control was determined using a MTT  
(3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium

bromide) assay which is commercially available (Sigma). Figure 30a shows representative data indicating that TPI 1396-34 significantly increased dose-dependent cytotoxicity of VP16, DOX, and TAXOL in DU145 prostate cancer cells. A more complete version of the data showing results at various concentrations of TPI 1396-34 is shown in Figure 31a. Similar results were obtained for PPC1 (Figure 31b) and PC3 (Figure 31c) prostate cancer cells treated with VP16, DOX, or TAXOL, and for H460 (Figure 31d) lung cancer cells treated with VP16 or DOX. Inactive poly-phenylureas compounds failed to sensitize tumor cells to anticancer drugs.

Similar tests of the effects of polyphenyl urea compounds on apoptosis induction by the biological agent TRAIL, an apoptosis inducing member of the Tumor Necrosis Factor (TNF) family were also performed. Cancer cell lines PPC1, ALVA31 and DU145 were treated with various concentrations of TRAIL alone or in combination with TPI 1396-34 at a concentration of 1  $\mu$ M. TPI 1396-34 sensitized PPC1, ALVA31, DU145, and HeLa cells to TRAIL-induced apoptosis (see Figure 30b). Inactive control compounds did not display this activity.

#### EXAMPLE XV

##### Polyphenylurea Compounds Demonstrate Anti-Tumor Activity in Clonogenic Survival Assays and Tumor Xenograft Models

This example shows that clonogenic survival of cancer cells is reduced by polyphenyl urea compounds. In addition, selected polyphenylurea compounds showed anti-

tumor activity *in vivo* using human tumor xenografts grown in immunocompromised mice.

In addition to short-term cytotoxicity assays, selected polyphenylurea compounds were tested for effects on clonogenic survival of cancer cells in colony formation assays, which can be considered a more stringent test of anticancer activity. Two prostate cancer cell lines PC-3 and LNCaP were cultured with TPI 1396-34 for 3 days, then culture medium was changed and colonies were counted one week later. Figure 32a shows the results obtained using various concentrations of TPI 1396-34 and one concentration (10  $\mu$ M) of a control compound. As seen in Figure 32a, TPI 1396-34 diminished clonogenic survival of these cancer lines in a concentration dependent manner, with an average  $EC_{50}$  dose of 3  $\mu$ M + 0.5  $\mu$ M (mean + std error). At a dose of 10  $\mu$ M, colony formation was reduced to < 5% of control, in contrast to inactive control compounds, which had relatively little effect.

Selected polyphenylurea compounds were tested for anti-tumor activity *in vivo*, using human tumor xenografts grown in immunocompromised mice. First, it was determined what doses of polyphenylurea compounds were tolerated by mice. It was found that 100 mg/kg delivered i.p. as a single or in divided doses resulted in no gross toxicity. For tumor xenograft studies, PPC1 prostate cancer cells (2.5 million) were injected subcutaneously into the flanks of 8 male Balb/C *nu-/nu-* mice. Half of the animals received i.p. injections of TPI 1396-34 in DMF (N,N-Dimethylformamide) at 30 mg/kg at day 7 and day 8, while the other half received DMF diluent

alone. Tumor growth was monitored at least twice weekly by external calipers (see Figure 32b). At 24 days after compound injections, mice were sacrificed and tumors were excised and weighed (see Figure 32b, inset). As can be  
5 seen in Figure 32b, treatment with TPI 1396-34 resulted in reduced tumor size compared to the DMF control.

Similar data was obtained using TPI 1396-22 (see Figure 33a and 33b). In these experiments PPC1 prostate cancer cells (2.5 million) were injected subcutaneously  
10 into the flanks of male Balb/C *nu-/nu-* mice. On days 5, 6, and 7, when tumors were about 125 mm<sup>3</sup>, mice were treated with 30 mg/kg of TPI 1396-22 or solvent by i.p. injection and tumor volume was measured by calipers at least twice weekly for 19 days after injection. In Figure  
15 33b, the mice were sacrificed at day 19 and the tumors excised and weighed.

Additional experiments were performed using a different tumor xenograft model. HCT-116 colon cancer cells (2.5 million) were injected subcutaneously in the  
20 flanks of female Balb/C *nu-/nu-* mice. On days 6, 7, and 8 when tumors were about 125 mm<sup>3</sup>, mice were treated with 30 mg/kg of TPI 1396-34 (n=10) or solvent control (n=19) by i.p. injection. Tumor volume was measured by external calipers at least twice weekly for 19 days (see Figure  
25 32c). On day 19, the mice were sacrificed and the tumors were excised and weighed. Again, tumor size was reduced in mice treated with TPI 1396-34 compared to solvent control.

In summary, dosing mice for just 2 or 3 days  
30 with TPI 1396-34 or TPI 1396-22 significantly slowed the

rate of growth of both PPC1 and HCT116 tumors, thus demonstrating *in vivo* anti-tumor activity of these chemical compounds.

5

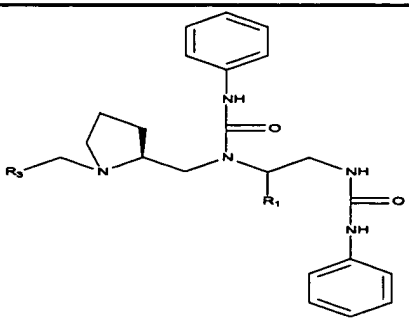
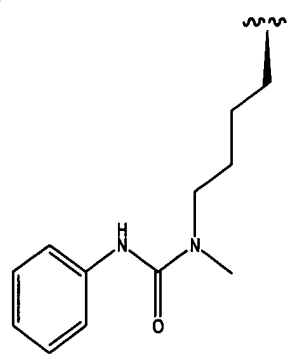
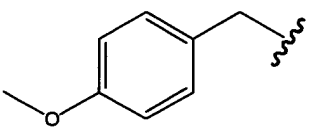
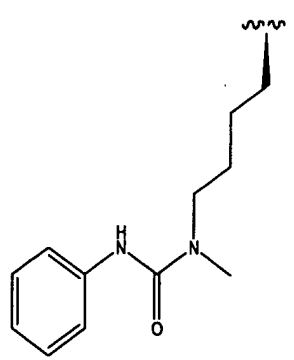
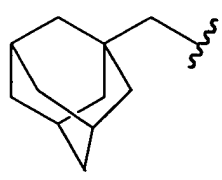
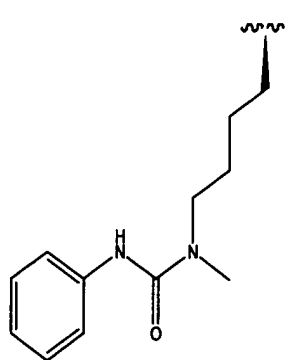
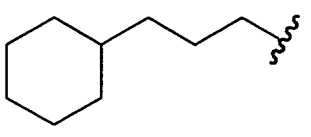
## EXAMPLE XVI

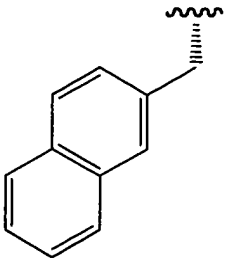
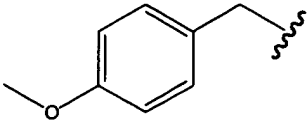
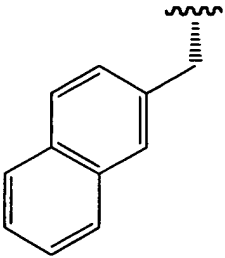
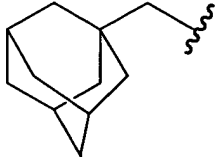
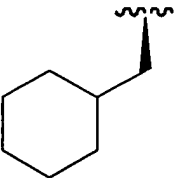
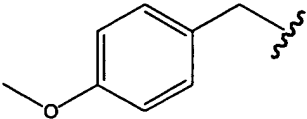
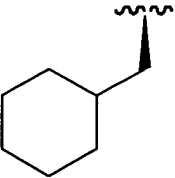
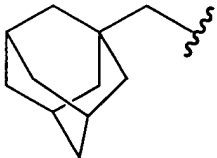
Structure Activity Relationship (SAR) of Individual TPI  
1396 Compounds and the Generation of TPI 1509 Compounds

This Example shows SAR information based on TPI 1396-1 through TPI 1396-36 (see Figure 22 for structures  
10 of these compounds) and the structure of TPI 1509 compounds. TPI 1509 compounds are based on on TPI 1396 compounds which are in turn based on the TPI 927 library. As shown by the activity screening of individual compounds TPI 1396-1 through TPI 1396-36, a number of different  
15 hydrophobic aromatic groups are acceptable for activity at the R1 and R3 positions (see Table X). Table X also shows that active compounds can be derived from a proline at the R2 position.



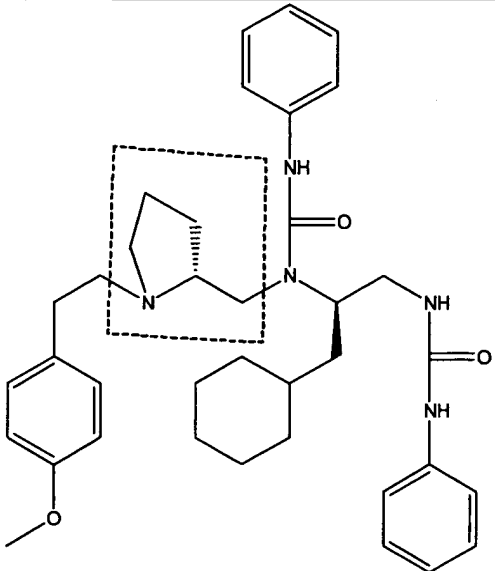
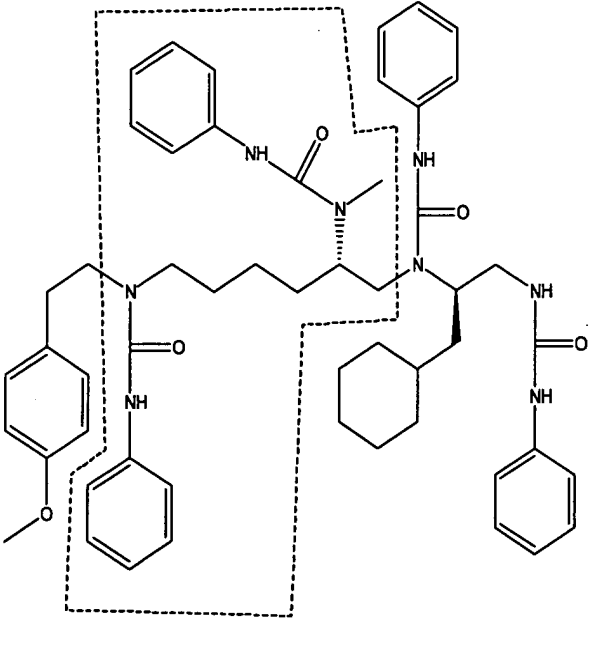
Table X

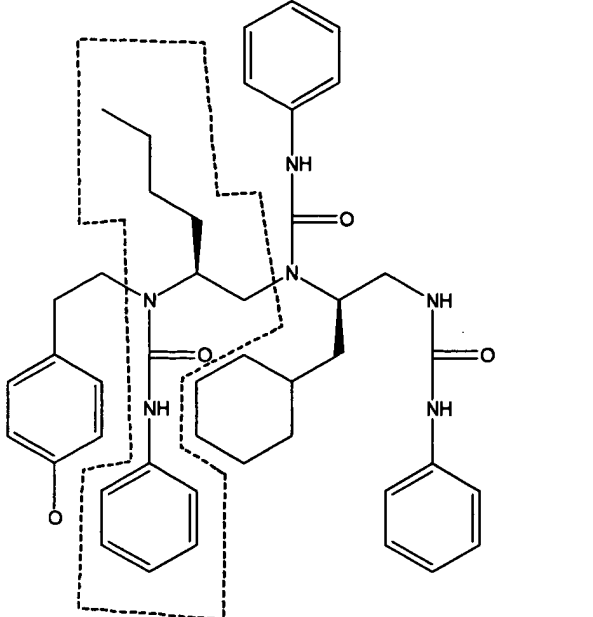
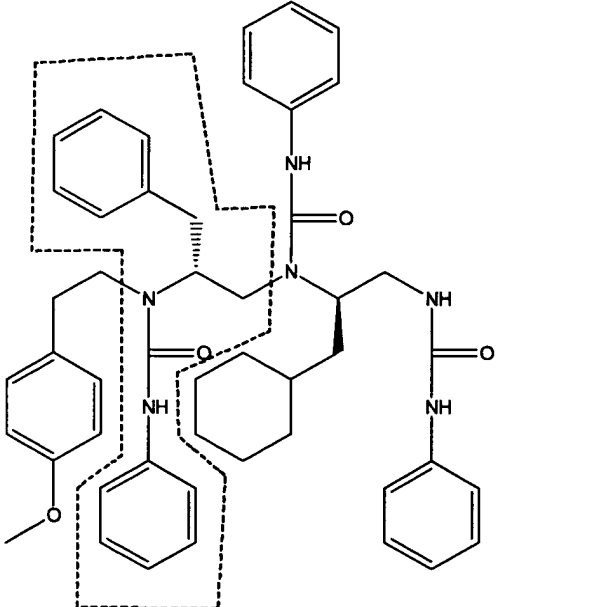
Core structure with R2 = L-Proline 			
TPI 1396 #	R1	R3	Activity*
10			2.8
11			2.6
12			2.7

22			3.3
23			2.6
34			3.0
35			2.5
5	*Relative caspase-3 activity in the XIAP derepression assay was calculated as the ratio of the Vmax in the presence of each compound divided by the Vmax of the controls having caspase-3 and XIAP.		

When examining individual compounds having different functionalities at the R2 position (Table XI), it can be seen that the diphenyl ureas derived from triamines having two secondary amines and one tertiary amine are more active than the triphenyl ureas. These diphenyl ureas were derived from the reduction of proline-containing acylated dipeptide amides, followed by treatment with phenyl isocyanate.

Table XI

TPI 1396-	Structure	Activity*
34		3.0
5 25		1.6

28		1.3
31		1.3
<p>*Relative caspase-3 activity in the XIAP derepression assay was calculated as the ratio of the V<sub>max</sub> in the presence of each compound divided by the V<sub>max</sub> of the controls having caspase-3 and XIAP.</p>		

Also, a series of compounds (TPI 1509, shown in Figure 34) synthesized using D-proline instead of L-proline were tested for activity. Figure 34 shows the names, structures and activity of these compounds in the XIAP derepression assay. All of these compounds were

active at 25 mg/ml, and the most active compounds in this assay were TPI 1509-1, TPI 1509-2, TPI 1509-3, and TPI 1509-6.

#### EXAMPLE XVII

##### 5 Structure Activity Relationship (SAR) of Polyphenylureas

This example demonstrates compounds that can be used to address the relative importance of the main scaffold and each R group on the activity of the poly-phenylurea compounds. Figure 35 shows compounds  
10 which are analogs of TPI 1509-7 where the properties of R1, R2 and R3 are varied separately by altering their chemical natures and therefore their physiochemical properties. A similar series of compounds was synthesized based on TPI 1396-34. The compounds are assayed for  
15 activity and another round of SAR can optionally be performed to further optimize the structure of the compounds. A desirable compound can be, for example, a compound with a lower molecule weight and better pharmacological properties than existing compounds.

20

#### EXAMPLE XVIII

##### TPI 1332 Peptide Compounds Interact with a Site on XIAP Distinct from the SMAC Binding Site

This example demonstrates that compounds in the TPI 1332 series of tetrapeptides do not interact with the  
25 SMAC binding site on XIAP.

Peptide compounds containing unnatural amino acids in the 792 series, 792-33 and 792-35 (see Figure 12

and 20 and Example XI), and an active compound in the TPI 1332 series, TPI 1332-69, were tested for binding activity in the SMAC binding assay as described above in Example X. For this assay, biotinylated SMAC 7-mer peptide (50 ng) was bound to 96 well plates coated with NeutrAvidin (Pierce, Rockford, IL) at 1 mg/ml in 100  $\mu$ l per well of 50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, 10 mM DTT. Then GST-XIAP was added at 0.1 mg/ml in 100  $\mu$ l with or without compounds in DMSO. After incubation for 1 hour at room temperature, plates were washed with PBS with 0.05% Tween 20, and bound GST-XIAP was detected by addition of mouse anti-GST monoclonal antibody (1:2000 dilution) followed by anti-mouse horse radish peroxidase conjugated IgG and 3,3',5, 5'-tetramethylbenzidine base (TMB) substrate with detection at 450 nm on a plate reader.

As shown in Figures 36 A-F and 37, the active compounds of the TPI 792 series and its analogs, as well as compounds of the TPI 1332 series, compete with XIAP binding to the SMAC peptide, with the exception of TPI 1332-69. In addition, as shown in Figure 37, while TPI 1332-69 is active in the derepression assay but does not compete for the SMAC peptide binding site, TPI 1495-5 (substitution analog of TPI 1332-69 with G at position 4) is active in the derepression assay and competes for the SMAC peptide binding site. As expected, the inactive compounds in the 792 or 1332 series do not compete with XIAP binding to the SMAC peptide.

As is shown in Figure 36C-F, many compounds of the 1332 series were capable of reversing XIAP-mediated suppression of caspase 3. Active compounds at 50  $\mu$ g/ml

(Figure 36C) included TPI 1332-1, -3, -4, -5, -11, -15, -32, -36, -38, -40, -41, -42, -45, -47, -63 to -69, -71 to -73, -76, -78, -81 to -85, -87 to -90 and -93. The activity of TPI 1332-1, -4, -41, -53, -69, and -77 was also determined in the derepression assay using XIAP-BIR2 domain, as shown in Figure 36E. The activity of TPI 1332-1, -2, -4, -6, -41, -47, -53, -55, -69, -76, -77 and -85, in the derepression assay using the cIAP1 BIR2 domain further is shown in Figure 36F. These data indicate that TPI 1332 and related compounds are active in derepressing caspase inhibited by XIAP or the BIR2 domain of XIAP, but do not overcome cIAP1-mediated suppression of caspase-3. It is important to note that the lack of activity observed for various compounds can be the result of the compounds being present at a two fold excess over cIAP1.

Shown in Figure 37 are the activities of compounds derived from TPI 1332-69, referred to as TPI 1495-1 (TPI 1332-69) and TPI 1495-2 through TPI 1495-9. Whereas TPI 1332-69 was active in the caspase derepression assay but inactive in the SMAC competition assay, TPI 1495-5 was active in both the caspase derepression assay and the SMAC competition assay. These data indicate that TPI 1495-5 has a binding site on XIAP that affects the functions of both the BIR2 domain and SMAC binding domain of XIAP.

Figure 43 shows several compounds derived from TPI 792-33 or TPI 792-35, referred to as the TPI 1453 series. As is shown, TPI 1453-1 is the same as TPI 792-33, with TPI 1453-2 through TPI 1453-5 being are modifications of TPI 792-33 that contain various natural and nonnatural amino acid substitutions; TPI 1453-5 is the

same as TPI 792-35, with TPI 1453-6 through TPI 1453-9 being are modifications of TPI 792-35 that contain various natural and nonnatural amino acid substitutions. Compounds TPI 1453-1, -2, -4, -6, -7, -8, and -9 were  
5 determined to have activity in both the caspase derepression assay and the SMAC competition assay.

These data indicate that a novel negative regulatory site on XIAP, the XIAP BIR2 domain, is not  
10 targeted by SMAC. Compounds that bind to this novel negative regulatory site such as TPI 1332-36, as well as compounds that modulate both the SMAC binding site and BIR2 domain, can be used in screening assay in order to identify other compounds that can bind to this novel site.  
15

#### EXAMPLE XIX

##### Identification of Compounds that Inhibit IAPs Other than XIAP

20 This example describes an assay that can be used to determine the effects of derepressors of XIAP-inhibited caspases on other IAPs.

Immunohistochemical analysis of prostate cancers indicates that cIAP1 and cIAP2 are commonly over-expressed  
25 in these tumors. Both cIAP1 and cIAP2 are caspase inhibitors (Roy, EMBO J. 16:6914-6925 (1997)) and they each bind SMAC (Du et al., Cell 102:33-42 (2000); Chai et al., Nature 406:855-862 (2000)). Moreover, molecular modeling studies indicate that some of the BIRs of cIAP1  
30 and cIAP2 are likely to bind SMAC, having great structural similarity to XIAP. These observations indicate that



derepressors of XIAP-inhibited caspases can have activity against caspases inhibited by these other IAPs.

To confirm that derepressors of XIAP-inhibited caspases have activity against caspases inhibited by these  
5 other IAPs the following assays are performed.  
Competition of the compounds with the SMAC peptide for binding to BIRs on XIAP is assayed. To accomplish this, the compounds are tested in SMAC competition assays in which FITC-conjugated SMAC tetrapeptide AVPI (SEQ ID NO:4)  
10 or FITC-conjugated HtrA2 tetra-peptide AVPS (SEQ ID NO:6) are bound to BIRs from XIAP. Rather than expressing full-length XIAP, fragments of XIAP containing only the BIR2 or BIR3 domains are expressed, as described in Takahashi et al., J. Biol. Chem. 273:7787-7790 (1998) and  
15 Deveraux et al., EMBO J. 17:2215-2223 (1998). These assays will determine if the compound functions as a SMAC-mimic, and also whether the compound targets BIR2 (the domain that inhibits caspases-3 and -7), BIR3 (the domain that inhibits caspase-9), both, or neither of these  
20 domains.

Additionally, enzyme depression assays are performed using BIR2 or BIR3 domains to pinpoint the domain in XIAP that is targeted by a compound.  
Recombinant purified BIR2 is mixed with caspase-3, and  
25 BIR3 with caspase-9, then the activity of these proteases is measured against specific fluorogenic substrate peptides (Ac-DEVD-AFC for caspase-3 versus Ac-LEHD-AFC for caspase-9) in the presence and absence of a compound in an effort to pinpoint whether the compound targets BIR2,  
30 BIR3, both or neither of these domains in the XIAP protein. These results can be used for structure-based

optimization of compounds using molecular modeling of the published structures of XIAP, BIR2 (Sun et al., Nature 401:818-821 (1999) and Riedl et al., Cell 104:791-800 (2001)), and BIR3 (Liu et al., Nature 408:1004-1008  
5 (2000)).

With respect to cIAP1 and cIAP2, similar enzyme derepression and SMAC competition assays are performed using full-length cIAP1 and cIAP2, as well as fragments containing individual BIR domains, thus determining  
10 whether the compounds cross-inhibit these other members of the IAP-family.

If a compound does inhibit cIAP1, cIAP2, or both of these proteins, then the potency of the compound can be improved through medicinal and combinatorial chemistry.  
15 Assays can be performed to contrast retention versus loss of cIAP1/cIAP2 activity *in vitro* with activity of compounds in cell-based assays. Structure activity relationship studies of this type indicate whether the optimal compound has selective specificity for XIAP versus  
20 pan-reactivity against several IAPs. The compounds with these different profiles (selective versus broad-spectrum activity) are contrasted with respect to toxicity issues, to obtain a compound with a desired balance between efficacy and safety.

25

## EXAMPLE XX

Mechanism of Action of PolyPhenyurea Compounds

To determine whether apoptosis induction by polyphenylurea compounds occurs through the intended

mechanism of action, toxicity of TPI 1396-34 was tested using cells obtained from XIAP knock-out mice in a cell based assay. Mechanism-based toxicity of TPI 1396-34 and daunorubicin on mouse embryo fibroblasts (MEFs) from XIAP  
5 -/- mice and transformed wild type (+/+) mice was determined. Cells were either tested directly at low passage (Figure 38 A and C) or after transformation by infection with a retrovirus encoding SV40 large T antigen (Figure 38 B and D). Cells were cultured 1 day with  
10 various concentrations of compound TPI 1396-34 (Figure 38 A and B) or with daunorubicin (Figure 38 C and D). Cell viability was measured by MTT assay, expressing data as a percentage relative to control, untreated cells. Data shown in Figure 38A-D represent mean  $\pm$  standard deviation  
15 of triplicate determinations.

These results demonstrate that XIAP-deficient cells are less sensitive to the polyphenylurea compound compared to wild-type MEFs, providing evidence that the compound functions through the intended mechanism of  
20 action since cells lacking the intended target (XIAP protein) are less sensitive. In contrast, if the compound induced apoptosis through a non-specific mechanism, it would be expected that XIAP-deficient cells would be more sensitive due to the absence of this anti-apoptotic  
25 protein. These findings also contrast non-transformed with transformed cells by showing that transformed cells are more sensitive to the XIAP antagonist. In contrast, conventional anticancer drugs such as daunorubicin do not display selectivity for transformed cells in these *in*  
30 *vitro* cytotoxicity assays.

## EXAMPLE XXI

Polyphenylurea Compounds Enhance Cytotoxicity of Antigen-Specific CTL

To determine if polyphenylurea compounds reduce resistance to apoptosis mechanisms relevant to CTL-mediated cell lysis, selected compounds were tested for their ability to enhance cytotoxicity of antigen-specific CTL.

For these experiments, tumor cells were loaded with  $^{51}\text{Cr}$  then pulsed with specific antigen and incubated with antigen-specific T cells at effector:target ratios of either 5 (Figure 39A) or 10 (Figure 39B) in the absence of compounds (open circles; dashed lines) or in the presence of 10  $\mu\text{M}$  of either inactive control compound TPI 1396-28 (squares) or active compound TPI 1396-34 (closed circles). After 4 hours,  $^{51}\text{Cr}$  release was measured. Data shown in Figure 39 are expressed as a percentage of total release induced by detergent lysis, and data represent mean  $\pm$  standard deviation of duplicate determinations.

As is shown in Figure 39, TPI 1396-34 sensitizes tumor targets to CTL-mediated lysis. These results provide evidence that polyphenylurea compounds are not deleterious to CTL effector function and indicate that inhibiting XIAP reduces resistance to apoptosis mechanisms relevant to CTL-mediated cell lysis.

## EXAMPLE XXII

In Vivo Activation of Caspases by Polyphenylurea Compounds  
in Tumors

To determine if polyphenylurea compounds induce  
5 caspase activation in tumors *in vivo*, TPI 1396-12 was  
tested a human tumor xenograft mouse model. For these  
studies, tumor-bearing Balb/c mice at 8 weeks of age were  
either injected i.p. for 3 successive days with 30 mg/kg  
of polyphenylurea compound TPI 1396-12 or with an equal  
10 volume of diluent (CNTL). Immunoblot analyses of tumor  
tissue, the results of which are shown in Figure 40A, were  
performed using an antibody specific for cleaved caspase-3  
or actin at 24 hours following the final injection of  
compound or control. These results indicate that  
15 polyphenylurea compound TPI 1396-12 induce caspase  
activation in tumors *in vivo*.

Figure 40B shows immunohistochemistry of tumor  
tissue sections using H& E stained sections (B and C);  
anti-caspase-3 antibodies and anti-PCNA antibodies (dark  
20 stained nuclei; D and E), anti-caspase-6 antibodies (dark  
staining; F and G) and anti-DFF40 antibodies (dark  
staining; H and I). As is shown by detection of surrogate  
marker PCNA, polyphenylurea compound TPI 1396-12 had no  
effect on tumor proliferation. These results provide  
25 further evidence that polyphenylurea compound TPI 1396-12  
functions through an apoptotic mechanism *in vivo*.

## EXAMPLE XXIII

In Vivo Toxicology Analysis of Polyphenylurea Compounds

To assess in vivo stability and toxicology of polyphenylurea compound TPI 1396-12, tumor-bearing mice were treated with doses of compounds previously determined to be adequate for achieving anti-tumor activity *in vivo* using xenograph model and toxicological analyses were performed. In these studies, Balb/c mice (8 weeks of age) were either untreated or injected i.p. for 3 successive days with 30 mg/kg of polyphenylurea compound TPI 1396-12, or with an equal volume of diluent (PBS containing 10% DMSO, 5% TWEEN 80). At 12 hours after the final injection, mice were sacrificed and blood was analyzed for white blood cell count (WBC), red blood cell count (RBC), and platelet count (PLT). Sera were assayed for BUN, bilirubin, ALT and AST. These data, shown in Figure 41, represent the mean  $\pm$  standard deviation for 3 mice. Although these data do not reach statistical significance, they indicate a trend.

Toxicology data shown in Figure 41 indicate that polyphenylurea compound TPI 1396-12 is not toxic at the administered dosage. In addition, histological analyses of tissues confirmed these results.

These and related studies indicated that polyphenylurea compound TPI 1396-12 has a maximum tolerated dose of greater than 200-400 mg/kg in mice (non-lethal); that anti-tumor activity was demonstrated with as little as two sequential daily 30 mg/kg i.p. doses; and

that polyphenylurea compound TPI 1396-12 is expected to be stable in human serum for greater than 48 hours.

#### EXAMPLE XXIV

##### Polyphenylurea Compounds Selectively Bind to BIR2

5           To obtain direct evidence that polyphenylurea compounds bind to the BIR2 domain of XIAP, NMR studies were performed. In these studies, polyphenylurea compounds TPI 1540-14 and TPI 1540-15 were shown to selectively bind to BIR2.

10            $T_{1\rho}$  measurements were formed at 200 ms with 400  $\mu$ M polyphenylurea compound TPI 1540-14, -15 or -20 in the absence and presence of 10  $\mu$ M GST-BIR2. Binding of active compounds TPI 1540-14 and TPI 1540-15 was manifested by a decrease in signal intensity in the presence of a sub-  
15 stoichiometric amount of GST-BIR2. Inactive compound TPI 1540-14 did not show this effect. As a control, an internal reference compound was added to the solution containing TPI 1540-15 (marked with a \* in Figure 42). As a control for compound binding to GST, the binding of TPI  
20 1540-15 was also tested against GST-Bcl-B, which produced a negative result. Results for TPI 1540-14 and TPI 1540-20 are shown in Figure 42. Results for TPI 1540-15 were similar to those observed for TPI 1540-14.

25           In summary, this example provides evidence that polyphenylurea compounds TPI 1540-14 and TPI 1540-15 bind directly to the BIR2 domain of XIAP.

## EXAMPLE XXV

Structure Activity Relationship (SAR) of TPI 1540  
Compounds

This example shows SAR information for  
5 individual TPI 1540 compounds TPI 1540-6 through TPI 1540-  
23. As is shown in Figure 35B, a number of different  
modifications can be made without altering activity in  
comparison to TPI 1509-7 or TPI 1396-34 as indicated by  
IC50 values observed in the XIAP derepression assay or  
10 Jurkat-Annexin V assay.

These SAR data show that structures of active  
compounds, such as TPI 1396-34 can be simplified while  
retaining complete activity against the target protein  
both *in vitro* and in cell based assays. In addition,  
15 these data show that polyphenylurea compounds can be  
optimized with respect to Lipinsky's rules and that,  
accordingly, pharmacological properties of selected  
polyphenylurea compounds can be optimized.

## EXAMPLE XXVI

20 Binding of BID and XIAP BIR2 domain to  
Biotinylated Peptides

This example describes competition assays for  
XIAP-BIR2 binding to biotinylated peptides of the TPI 792,  
TPI 1332 and TPI 1495 series. The biotinylated peptides  
25 are referred to as the TPI 1554 series, and include TPI  
1554-1 through TPI 1554-8, as shown in Figure 44. Also  
shown in the table of Figure 44 is the TPI number



corresponding to the original tetrapeptides (Non-biotin Synthesis #) and the molecular weights (MW).

Binding assays were performed to determine  
5 whether the biotinylated peptides bound to the BIR domain  
of XIAP. Biotinylated peptides were adsorbed to  
Neutravidin-coated plates, and GST-XIAP BIR2 or GST-BID  
(control) was added. Bound GST polypeptides were detected  
10 with an anti-GST antibody. Figures 45A-J show that  
biotinylated compounds TPI 1453-1 (TPI 1554-1) (A); TPI  
1453-6 (TPI 1554-2) (B); TPI 1332-4 (TPI 1554-3) (C); TPI  
1332-41 (TPI 1554-4) (D); TPI 1332-69 (TPI 1554-5) (E); TPI  
1332-77 (TPI 1554-6) (F); TPI 1495-19 (TPI 1554-7) (G), and  
TPI 1495-20 (TPI 1554-8) (H) competed for binding to XIAP-  
15 BIR2 to an extent comparable to the binding of SMAC  
peptides SMAC 7-mer (I) and SMAC 4-mer (J).

Figures 46 A-C show binding of XIAP-BIR2 to  
biotinylated tetrapeptides TPI 1554-1 through TPI 1554-8  
20 using 1 µg/ml XIAP BIR2 (A); 0.5 µg/ml XIAP BIR2 (B), and  
0.25 µg/ml XIAP BIR2 (C) with biotinylated peptides at  
concentrations of 1.6 µg/ml, 0.4 µg/ml and 0.1 µg/ml.  
TPI 1554-3 through TPI 1554-8 had a greater extent of  
binding to XIAP BIR2 domain in comparison to TPI 1554-1  
25 and -2. These results show that binding of XIAP BIR  
domain to biotinylated tetrapeptides TPI 1554-3 through  
TPI 1554-8 is comparable to the binding of SMAC peptides.

Figure 47 shows competition assays for the  
30 binding of XIAP BIR2 domain to the biotinylated  
tetrapeptides in the absence or presence of a  
polyphenylurea compound, using biotinylated peptide TPI  
1554-5 (TPI 1332-69) (A), which is a non-SMAC mimic; and

biotinylated peptide TPI 1554-3 (TPI 1332-4)(B), which is a SMAC mimic. Results from these studies indicate that the polyphenylureas do not compete for binding of XIAP-BIR2 domain with either of the tetrapeptides. However, 5 the SMAC mimic tetrapeptide TPI 1554-3 competes with binding of XIAP BIR2 domain to the biotinylated non-SMAC mimic tetrapeptide TPI 1554-5. This observation for TPI 1554-3 is consistent with the finding described herein in Example XVIII that TPI 1332-69 binds to a site on XIAP 10 that overlaps between the SMAC and BIR2 binding sites.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated 15 by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the examples provided above, it should be 20 understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.